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IgTCR-Modified T Cells for Breast Cancer Therapy: Phase I Studies.
T cells are capable of penetrating any biologic space and eliminating cells displaying foreign antigens. We have used a chimeric IgTCR molecule to create T cells with antigen-specificity directed against the CEA tumor antigen. In the present study we have examined the toxicity and tolerability of autologous T cells modified with this receptor in CEA+ breast cancer patients. Patients have tolerated the treatment well, and no adverse events could be attributed to the therapy. Preliminary efficacy data suggest that IgTCR-modified T cells may have some anti-tumor activity in vivo, but are limited by poor circulation after infusion. Research aimed at improving circulation have focused on isolating/expanding subpopulations of T cells with a memory cell-like phenotype. These cells enter a resting state when deprived of IL2 rather than a apoptosis pathway. These memory-like cells may persist and circulate much better than standard ex vivo expanded cells. We have also shown that CD28 co-stimulation signals are required to induce proliferation of IgTCR+ T cells. Incorporating antigen-driven T cell proliferation into the activation pathway should allow any T cells that do infiltrate tumor to proliferate rapidly to high numbers. Together, these results suggest that IgTCR-modified autologous T cells may become an important modality for the treatment of metastatic breast cancer.

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5. INTRODUCTION

Carcinoembryonic antigen (CEA) is a tumor-associated antigen which is expressed on ≈30-60% of metastatic breast tumors. The purpose of this research is to develop a new type of cancer therapy using autologous T cells modified with a chimeric immunoglobulin T cell receptor (IgTCR) directed against CEA+ tumors. The specific objectives are to:

1. Complete setup for therapy.
2. Apply IgTCR-modified cells in a phase I clinical study in patients with metastatic CEA+ breast tumors
    a. Determine the safety and tolerability of anti-CEA modified T cells.
    b. Describe the pharmacokinetics by the persistence of modified T cells in blood.
    c. Evaluate immunogenicity of the modified cells.
    d. Measure immunologic and other parameters which correlate with efficacy.
    e. Preliminary evaluation of efficacy.

In addition, to the above, we have also carried out basic research efforts to create second-generation reagents that will enhance the therapy for future phase II/III studies.

6. BODY:

I. Complete setup for therapy

A. Administrative/Management.

Final Institutional Review Board (IRB) approval for the proposed study was received September 8, 1998. An IND was filed on 8/27/97 and was removed from clinical hold in April of 1998. Other approvals previously reported but subsequently modified or amended include final authorization to use the General Clinical Research Center at the BIDMC and the release of funds for the production of clinical grade retrovirus at the NIH-sponsored National Gene Vector Laboratories (NGVL).

Construction of the vector producer cell line to be used to manufacture virus was carried out at the NGVL with on- and offsite supervision by members of the Biotherapeutics Development Lab (BDL). The final testing (sterility, safety, identity and potency) of the vector producer cell line was carried out at both the NGVL and BDL and was completed in March of 1998. Clinical grade virus was manufactured and tested for sterility, purity, and potency (at NGVL and BDL) in March-May, 1998. Clinical grade retrovirus was released and shipped to the BDL in May, 1998.

B. Setup of Transduction Facility.

Optimization and standardization of the T cell transduction and expansion protocols was carried out in the production facility at the BDL in April-May, 1998. This involved testing of all ancillary reagents, as well as mock production runs to test various components of the Fenwal culture and harvesting system, with which we had no prior experience. Final filing

of the Standard Operating Procedures (SOPs) covering the use of the facility was completed at the BDL by the end of May, 1998. The facility became fully functional in June, 1998, when the first production of gene-modified T cell was conducted out for a phase I study similar to the present anti-CEA breast cancer trial. Manufacturing and testing of T cells for the first breast cancer patient began in August, 1998.

II. Phase I Study.

A. Preparing IgTCR-modified T cells for patient treatment

Autologous IgTCR-modified T cells from patient blood was prepared by activating T cells with OKT3 for 2-3 days and then infecting the cells with high titer IgTCR retroviral supernate. Representative results on IgTCR expression and function in T cells from patients treated are shown in Fig.1. The IgTCR transgene was expressed at variable levels in 30-50% of the T cells used for actual treatments (Fig.1a). On average, $\approx$60% of these IgTCR-expressing cells were CD4+ and $\approx$40% were CD8+ (Fig.1b). *In vitro* tumor cell killing assays performed on patient cells prior to infusion demonstrated potent, CEA-specific anti-tumor activity (Fig.1c). To prepare actual doses used for treatment, patient T cells were expanded in culture bags to the levels required for each dose. We have expanded patient T cells to levels as high as $4x10^{11}$ total cells, although we have not yet treated any breast cancer patients at these higher dose levels. While our initial expansions of large numbers of T cells required a significant effort, we have subsequently refined our manufacturing procedure such that we can now routinely prepare $1x10^{11}$ cells (maximum dose level) in about 12-14 days. Together, these results demonstrate our ability to rapidly prepare large doses of clinical grade, IgTCR-modified T cells, while maintaining a high transduction efficiency (>30%), high viability (>90% viability) and potent anti-CEA activity.

B. Toxicity, Tolerability, and Maximum Tolerated Dose

Thus far, two breast cancer patients in the low dose cohort (four doses, escalating from $1x10^9$, to $3x10^9$, $1x10^{10}$, and $3x10^{10}$) have been treated. In approximately 20% of the doses, a low grade fever followed the infusion. This was attributed to the co-administration of IL2 (75,000 IU/Kg/day) which is delivered through a continuous infusion pump. Infusions of IgTCR-modified T cells were well tolerated by both patients and no serious adverse events could be attributed to the treatment. The second patient died of her disease prior to receiving her final planned dose of $3x10^{10}$ cells.

With only two patients treated, it is too early for an assessment of the maximum tolerated dose (MTD), or the overall toxicity of the treatment. We anticipate that the MTD will be $1x10^{11}$ cells based on our results in a closely related study in patients with CEA+ colon carcinomas. This study utilizes the same IgTCR construct as the breast cancer study but does not include co-administration of low dose IL2. The colon carcinoma study has shown no dose limiting toxicity, and the optimum biologic dose (OBD) is $1x10^{11}$ cells, which is our maximum practical dose (MPD). Based on these results, we would predict that no dose limiting toxicity from the IgTCR cells will be obtained in the breast cancer trial. However, we will need to complete the treatment of the breast cancer patients in the high dose cohort to determine if the OBD will be significantly affected by co-administration of IL2. Treatment of the final four patients in the breast cancer trial will be completed in year two of

the grant.

C. Pharmacokinetics

Circulating levels of IgTCR+ T cells were followed by harvesting blood samples from patients before, and at various times after infusion, purifying lymphocytes by centrifugation over Ficoll, staining the purified lymphocytes with anti-idiotype antibody and assaying the cells by FACS. At all doses tested thus far, we have not detected any IgTCR+ cells in patient blood samples. The rapid disappearance (< 1 hour) of T cells infused into patients demonstrates that the infused T cells leave the pool of circulating lymphocytes almost immediately. This is similar to the findings of previous studies which showed T cells localizing to the lungs, liver and spleen (1-3). These same studies also showed eventual localization of T cells to tumor deposits, although the number of infiltrating cells was small relative to the total number of cells infused. Nevertheless, larger doses of T cells ($\approx 1x10^{11}$) in the TIL studies were associated with an enhanced likelihood of anti-tumor activity in vivo, even without high fractions of cells redistributing to tumor (ref. 4 and J. Yannelli; pers. comm.). This is our current conceptual model. Means to improve local T cell proliferation and infiltration and their relevance to the original hypothesis are discussed below.

Despite our inability to detect IgTCR+ T cells in standard preparations of whole blood, we have been able to detect IgTCR+ T cells through a modified pharmacokinetic assay. In this assay, preparations of patient blood are reactivated prior to FACS to enrich for T cells. This involves taking an aliquot of each blood sample and activating with OKT3 and IL2. The cells are then cultured in T cell growth media for $\approx$7 days. During this time, any T cells in the blood sample rapidly proliferate while other cell types die out. These activated cultures are then stained with anti-idiotype antibody and assayed by FACS. In blood samples obtained from patients in the low dose cohorts, $\approx$1-5% of the T cells in the cultures are IgTCR+. In blood samples obtained from patients in high dose cohorts, as many as 20% of the cultured cells were IgTCR+. For most of the doses infused, the total number of IgTCR+ T cells in circulation peaks around one day after infusion. In most cases, IgTCR+ T cells can be recovered from blood samples, albeit at lower levels, 2-3 days after the infusion. In one instance, IgTCR+ T cells could be detected a late as 7 days after the infusion. These studies demonstrate that the infused T cells do circulate in the blood at very low levels, and that these circulating IgTCR+ cells are viable.

One problem with this type of detection method is that IgTCR+ T cells in patient blood samples have been previously activated with OKT3 and it is possible that this previous activation provides them with either a growth advantage, or disadvantage when they are reactivated a second time in patient blood cultures. This could lead to either an over- or underestimate of the actual numbers of IgTCR+ cells in the blood. Studies were conducted to validate the modified pharmacokinetics assay by analyzing control blood samples into which a known proportion of IgTCR+ T cells had been added. This was done with blood samples that were subsequently OKT3 activated and cultured for 7 days prior to FACS analysis, as well as, with Ficoll-purified blood which was not activated prior to analysis. Experiments using the OKT3 reactivated cultures showed that IgTCR-modified T cells in reactivated cultures have a slight growth advantage (3-4 fold on average) when very low numbers of IgTCR+ cells are added (when 1-10% of cells added were IgTCR+), but no

growth advantage when higher proportions of IgTCR+ cells were added (>10%) (data not shown). These results suggest that the modified pharmacokinetics assay may slightly overestimate the number of IgTCR+ cells that are circulating in the blood, particularly when the number of IgTCR+ cells is low.

Experiments using samples in which known proportions of IgTCR+ cells were added to Ficoll-purified blood and then assayed immediately showed two interesting results. First, only about 20-40% of the added IgTCR+ T cells could be actually detected by flow cytometry. However, even when very low proportions of IgTCR+ cells were added (0.5%) it was still possible to detect a fraction of the cells by FACS. These results suggest two aspects regarding the presence of IgTCR+ cells in patient blood. The first is that IgTCR+ T cells are present at low frequency in blood samples, but are masked by the high background signals caused by various other cell types present in the blood preparations.

More interestingly, the results suggest that the IgTCR+ cells that are present in blood samples are morphologically different than the cultured T cells added to the validation assays. This conclusion is based on the observation that the proportion of IgTCR+ T cells that can be recovered from patient blood (by reactivation) is higher (1-20%) than the lowest proportion of IgTCR+ cells that can be detected ($\approx$0.1%) when IgTCR+ cells are artificially added to a blood sample. In the absence of a growth advantage, the IgTCR+ cells present in patient blood must reside in a different position on the forward scatter-side scatter plots used to gate the flow cytometer on the white cell component of blood. One possible explanation for this is that the IgTCR+ cells present in patient blood represent a distinct sub-population of T cells with a morphology that allows them to remain in circulation, and be recovered when patient blood samples are reactivated.

Although several details of the modified phamacokinetic assay still need further investigation, we have shown that a FACS-based assay system can be used to measure the pharmacokinetics of gene-modified T cells in vivo. Very few methods exist for the detection of ex vivo-activated T cells after they are infused into patients. The FACS-based assay system we have developed is rapid, amenable to a detailed analysis of T cell subsets, and has given consistent results from patient to patient. To our knowledge, the utility of this type of methodology has not been previously demonstrated.

Unfortunately, the development of this modified pharmacokinetics assay was not completed prior to the treatment of the first two breast cancer patients, and, as such, we can not yet provide a detailed pharmacokinetics analysis for the breast cancer study. We have however done this analysis in the parallel colon carcinoma study and these results are shown in Fig.2. We provide this data only to demonstrate the nature of the pharmacokinetics data we expect to obtain when additional breast cancer patients have been treated. Shown in the graph are the number of IgTCR+ T cells actually detected relative to the total number of IgTCR+ cells that were infused over the course of four doses. Over the course of the treatment, the pharmacokinetics of the infused T cells resembled the sawtoothed curves characteristic of multidose regimens with drugs that are rapidly cleared from the circulation. The results clearly show that the majority of the infused cells rapidly leave the pool of circulating lymphocytes. One important aspect of the breast cancer study is the co-administration of

IL2. This IL2 should prolong the survival of IgTCR+ cells in vivo. Our previous results in the colon carcinoma study showed that, in the high dose cohort, IgTCR+ cells could occasionally be detected as long as 7 days after infusion. The co-administration of IL2 may prolong the survival of IgTCR+ cells in vivo, and by extension, prolong their circulation. This will be evaluated in a detailed pharmacokinetic analysis when the remaining breast cancer patients are treated in year two of the study.

D. Pharmacodynamics

1. Localization- If biopsy tissues can be obtained from the remaining four patients, we will perform immunohistochemistry to determine if IgTCR+ cells are infiltrating tumor tissues.

2. Production of soluble factors. Frozen serum samples for each dose and post-infusion time point have been collected for analysis of cytokine production and levels of soluble Tac. Data on cytokine and soluble Tac levels are not yet available.

3. Immunogenicity. Of the two patients treated thus far, neither showed any indications of an immune response against the vector. Testing of serum samples from one patient for antibodies directed against the humanized MN14 antibody fragment used in the IgTCR construct was negative.

E. Laboratory Studies.

Lab studies were performed to evaluate cell quality and safety, as well as to monitor peripheral blood for any imbalances induced by the therapy.

1. Quality Control. Each dose of IgTCR-modified T cells that was administered was analyzed for sterility by the following methods; bacteria/fungus (standard microbiologic tests), endotoxin (limulus amebocyte lysate assay), replication competent retrovirus (S+L- assay with amplification), and mycoplasma (Mycotect, Gibco/BRL). All doses administered were negative for each of the above tests in both breast cancer patients that have been treated so far.

2. Lab correlates. Blood samples from patients were analyzed for alterations in T cell subsets and for the presence of IL2 receptor (Tac+) cells by flow cytometry. Expression of the IL2 receptor is increased in activated T cells. Neither breast cancer patient showed any significant deviation in the proportions of individual T cell subsets. In both patients, the numbers of Tac+ cells was increased in the later doses relative to the first dose. The number of Tac+ cells was also higher in breast cancer patients relative to the colon carcinoma patients treated. These results may be due to the low dose IL2 which was co-administered in the breast cancer trial. In both cases however, the elevation in Tac+ cells was slight and may not be statistically significant.

F. Preliminary evaluation of efficacy.

With only two patients treated at the lowest doses, it is difficult to evaluate efficacy at this time. However, there are some signs that the treatments will lead to anti-tumor responses. Previous studies using TILs have shown that higher doses of T cells ($\approx 1x10^{11}$) are associated with an enhanced likelihood of anti-tumor activity in vivo (4 and J. Yannelli; pers.

comm.). No breast cancer patients have yet been treated at the $1x10^{11}$ dose. However, in our parallel colon carcinoma trial, two patients have been treated at this dose level. One of these patients has shown objective indications of an anti-tumor response. In this patient, levels of soluble CEA declined by 50% (Fig.3) following infusion of T cells. Further, this decrease in soluble CEA was accompanied by the alleviation of pain symptoms sufficient enough to allow cessation of narcotics administration. Interestingly, this patient also showed the highest levels of circulating IgTCR-modified T cells, suggesting that effective anti-tumor responses are strongly influenced by the mobility of the infused cells. It is likely that doses of $1x10^{11}$ cells used in combination with IL2 in the breast cancer study will allow us to built on these early promising results.

III. New research on second-generation reagents

As indicated above, ex vivo-modified T cells do not infiltrate tumor deposits with high efficiency. Overcoming this specific obstacle is likely to be a significant breakthrough since, anti-CEA IgTCR-modified T cells are exceptionally proficient at killing once contact with tumor cells occurs (5). Based on our assessment of the mechanisms that actually limit the efficient trafficking of modified T cells in vivo, we have focused our basic research efforts in two major areas. These areas are:

1. Inducing IgTCR+ cells to undergo a rapid exponential expansion after contacting CEA.
2. Developing methods to improve the circulation of T cells grown ex vivo.

1. Inducing T cells to undergo a rapid exponential expansion after contacting CEA.
The ability of IgTCR-modified T cells to effect tumor reduction in vivo may be reflected in the degree to which IgTCR-induced immunity mimics natural immunity. the primary hypothesis underlying IgTCR immunity is that crosslinking of the chimeric receptor alone, will be sufficient to activate all T cell effector functions. This is supported by numerous studies (6-18) showing that crosslinking the IgTCR induces potent CTL activity and cytokine release. These studies have not however, shown that crosslinking the IgTCR induces a logarithmic, antigen-driven expansion of IgTCR+ T cells. This distinction is important because, in the absence of antigen-induced T cell proliferation, the small numbers of T cells that actually infiltrate tumor deposits may be insufficient to cause tumor reduction.

One important result of our basic research efforts was the demonstration that IgTCR-modified T cells do not proliferate after binding the target antigen unless the T cells also receive CD28 co-stimulation signals (manuscript in preparation). Antigen-induced proliferation is a crucial component in generating a self-sustaining immune response. As shown in Fig.4, coupling the primary activation signal from the IgTCR molecule with a secondary CD28-mediated signal induces a dramatic polyclonal expansion of IgTCR+ T cells. Accordingly, we are currently testing second generation vectors that are designed to incorporate CD28 co-stimulation into the engineered activation pathway. This is accomplished by integrating anti-CEA-IgCD28 chimeric constructs into our anti-CEA IgTCR clinical vectors. These constructs couple the same anti-CEA antibody fragment used in our IgTCR constructs to the signalling portion of the CD28 receptor (19, 20). We have shown

that both the IgTCR and IgCD28 gene products are properly processed and expressed on the cell surface (Fig. 5). We are currently testing the ability of these dual-chimera vectors to induce CEA-specific CTL activity, cytokine release, and CEA-driven polyclonal expansions of PBMC-derived T cells.

The use of CD28 co-stimulation was anticipated in our original application to be incorporated into a follow up phase II" study if earlier applications do not meet efficacy criteria (see original application). This was originally proposed to involve co-administering anti-CD28 antibodies, rather than a second chimeric construct. We are evaluating the relative merits of CD28 antibody (21) (under discussion with the FDA for human use) versus incorporating CD28-signalling into the retroviral vector through an anti-CEA-IgCD28 chimera (19, 20).

2. Developing methods to improve the circulation of T cells grown ex vivo.

We have hypothesized that the poor circulation of ex vivo-modified T cells is due to the initial OKT3 activation of the cells and its associated upregulation of adhesion factors. This initial activation step is required in order to transduce the cells and cannot be eliminated. However, deactivating the T cells prior to infusion may reduce the expression of these adhesion factors and significantly improve T cell circulation and tumor infiltration. To accomplish this, we have focused on developing a culture system that will allow patient T cells to be deactivated prior to infusion.

Ex vivo-modified T cells are grown by initially activating the cells by crosslinking the T cell receptor (TCR) with OKT3 (anti-TCR antibody). Following TCR stimulation, the activation state of the cells is maintained solely by the presence, or absence of IL2. In the presence of IL2 T cells remain highly activated, while if IL2 is withdrawn, the cells deactivate and enter an apoptosis pathway. IL2-dependent deactivation is associated with morphological changes that suggest adhesion factors are downregulated. Deactivated cells convert from large cells that grow in grape-like clusters to small, rounded cells that are dissociated into single cell suspensions.

Unfortunately, IL2-resting also leads to the massive death of T cells (5), which is not compatible with growing large numbers of T cells for infusions. We observed however, that a small proportion of the T cells ($<1\%$) remained viable for long periods of time in the absence of IL2. Initial experiments with this subpopulation of surviving T cells have shown that they retain the ability to bind CEA and specifically lyse CEA+ tumor cells (5). We have subsequently shown that these IL2-rested T cells are able to reactivate when exposed to antigen plus IL2, and with the appropriate co-stimulation signals (discussed above), will rapidly proliferate to high numbers (Fig.6). Furthermore, re-expanding these IL2-rested T cells leads to a population of cells that do not enter an apoptosis pathway when deprived of IL2 a second time (Fig.6). These re-expanded T cells have been carried for 30 days in the absence of IL2 without any significant decline in viability (Fig.6).

Phenotypically, these T cells resemble memory cells since they can remain viable for long periods of time without IL2, and retain to ability to reactivate and proliferate when re-exposed to antigen plus IL2. These memory cell-like characteristics give these cells several

advantages over ex vivo-modified T cell populations currently used in adoptive immuno-gene therapy approaches.

These advantages are outlined below:

1. Memory-like cells expanded using OKT3-stimulation retain expression of the IgTCR gene (Fig.7B-C), allowing them to be used with the currently achieved transduction efficiencies ($\approx$30-50% IgTCR+ cells).

2. Memory-like cells can also be generated by stimulation through the chimeric receptor (data not shown). This leads to the selective proliferation of only the IgTCR+ cells, and the conversion of a mixed population of cells to one that is 100% IgTCR+ (Fig.7D-E). The ability to rapidly manufacture T cell preparations that are 100% positive will eliminate a significant source of variation from patient to patient.

3. Because these memory-like cells retain viability better than standard preparations of ex vivo-modified cells, they are much more durable. This makes it easier to maintain and control large-scale expansions once a culture has been established. This will greatly simplify the manufacturing process.

4. Because these memory cells are long-lived and can enter a resting state, they may persist for longer periods of time, and circulate in higher numbers in vivo. We are currently designing experiments to directly address this in animal models. More efficient circulation of IgTCR-modified cells may lead to higher levels of tumor infiltration and better anti-tumor responses.

5. Because these memory-like cells can reactivate and proliferate on re-exposure to antigen, it may be possible to use fewer cells/dose. This would greatly reduce the costs of this type of therapy and simplify the manufacturing process.

IV. Summary.

The research conducted under year one of the grant has shown our ability to prepare and administer large numbers of IgTCR-modified patient T cells (CD4 and CD8). This includes the documentation of drug safety, sterility, and potency for individual patients. Preliminary results on efficacy coupled with the lack of toxic side effects *in vivo* suggest that this therapy may ultimately become an important modality for the treatment of CEA+ breast cancer. However, obtaining unequivocal anti-tumor responses that directly reduce morbidity and mortality from metastatic breast cancer may require additional refinements.

Our pharmacokinetic studies indicate that the modified T cells do not circulate well after infusion and this may limit their ability to be distributed into tumor beds. Efficient delivery of gene-modified T cells into tumor cell deposits may be a crucial step in achieving effective tumor reduction. We have developed preclinical data to support a two-pronged approach to solving this problem. This involves on one hand, imparting greater mobility and durability to the gene-modified T cells by selectively expanding rare memory cell-like clones, and on the other hand, providing for antigen-induced expansions of IgTCR+ T cells that come in

contact with tumor cells in vivo. Our results indicate that memory-like T cells can be isolated and selectively expanded from normal blood. Further, we have shown that incorporating CD28 signalling is sufficient to induce a rapid and sustained T cell proliferative burst.

Our plans for year two of the grant are to complete the phase I study. Because of the high costs, three patients in cohort 2 will be treated with one dose of $1x10^{11}$ cells instead of four doses, then closing the study. In the post funding period we plan to initiate clinical testing of T cells modified with dual chimera vectors using both a standard protocol for ex vivo expansion and a protocol that specifically selects cells that will persist for longer periods of time in vivo.

Key Research Accomplishments

- Completion of set-up for therapy
  - Safety, Sterility, Identity, and Potency testing of vector producer cells
  - Safety, Sterility, and Potency testing of retroviral supernate
  - Testing of ancillary materials
  - Establishment of T cell transduction Facility at Harvard Institutes of Medicine
  - Submission of Investigational New Drug application with FDA

- Initiation of Phase I testing in patients with metastatic breast cancer.

- Successful expansion of large numbers ($>4x10^{11}$) of clinical grade, ex vivo-modified human T cells.

- Development of a FACs-based assay for measuring the pharmacokinetics of gene-modified T cells in vivo.

- Generation of data that will allow for the submission of four peer reviewed manuscripts

- Discovery of a new type of retroviral vector that increases viral titers by 100-1000 fold

Reportable Outcomes

- Manuscript: Dynamics of tumor cell killing by IgTCR Modified T cells
Beecham et. al. 1999, Accepted, *J of Immunotherapy*
see Appendix B

- Manuscript: T cell proliferation induced by IgTCR and CD28 signals.
Beecham et al., in preparation

-Manuscript: In vitro isolation and expansion of T cells with a memory cell-like phenotype from normal PBMCs.
Beecham et. al., in preparation

-Manuscript: Development of a new class of retroviral vectors that allow selection for either viral titers of $>1x10^8$, or for high expression in target cells
Beecham et. al., in preparation

-Patent: New class of Retroviral Vectors: Reagents and methods for high efficiency viral gene transfer
submitted to U.S. Patent office, May, 1999

Conclusions

The present research has shown that normal T cells from breast cancer patients can be genetically programmed to respond against CEA+ tumor cells. This is demonstrated by the induction of potent CEA-specific cytolytic activity in patient T cells. The high CEA-specificity of gene-modified T cells is reflected in the low in vivo toxicity of IgTCR-modified T cells. Although we cannot yet claim major anti-tumor responses, we have observed objective indicators of response in at least one patient treated. However, our pharmacologic data indicates that poor T cell circulation is a general problem with this type of therapy. Poor circulation of modified cells may significantly limit the ability of T cells to distribute into tumor tissues. The importance of T cell circulation is supported by our finding that the single responding patient had significantly higher levels of circulating cells. Thus, although these gene-modified T cells may be capable of mediating tumor reduction in vivo, inefficient circulation limits the delivery of T cells into tumor tissues. In previous studies (4), similar inefficiencies in circulation have necessitated the use of large numbers ($>1x10^{11}$) of T cells to obtain measurable anti-tumor responses.

We have hypothesized that the poor circulation of gene-modified cells results from physiological changes that occur when T cells are activated for ex vivo expansion. These primarily involve increased expression of adhesion factors caused by the initial activation of the cells with OKT3 and IL2. The high level of adhesion factors on the surface of the cells causes gene-modified cells to lodge in the first high endothelial venule that they encounter after infusion, namely those in the lung. Although this delivery barrier does not completely invalidate our original hypothesis (that IgTCR-modified T cells will mediate tumor regression) it does demonstrate that modified approaches will need to be implemented in order to obtain marked tumor reduction.

We have focused our efforts at improving the therapy on two new approaches. The first approach is designed to increase the proportion of circulating cells. To accomplish this, we have developed a technique that allows for the isolation of rare, memory-like cells from the starting pool of IgTCR+ cells. These memory-like cells are capable of surviving for long periods of time without IL2 and retain the ability to kill CEA+ tumor cells (5). Further, in the absence of IL2, these cells enter a resting state that is associated with a decrease in cell-to-cell adhesion. The use of these cells for therapy should improve T cell circulation, lengthen the persistence of IgTCR+ cells in vivo, and improve the cost effectiveness of the treatment.

The second approach is designed to increase the potency of those cells that currently are able to circulate and infiltrate tumor tissues. This approach uses CD28 co-stimulation to endow IgTCR+ cells with the ability to rapidly proliferate in response to binding the target antigen. A rapid and prolonged proliferative burst will allow any IgTCR+ cells that do infiltrate tumor tissues to amplify in situ. This amplification at the cellular level should lead to a concomitant amplification of tumor cell killing and improved tumor reduction.

The final element for improving the therapy is the availability of IL2. Completing the present study will allow us to determine the effectiveness of low dose IL2 and high dose

IgTCR co-administration. It is possible that the administration of low dose IL2 will lead to better responses with our current methods of preparing modified T cells. If low dose IL2, as currently administered, does not lead to improved responses, we will undertake methods to increase the availability of IL2 in vivo. This can be accomplished simply by increasing the amount of IL2 administered. However, systemic administration of high dose IL2 can cause significant side effects. As such, we are currently investigating if the full activation of IgTCR-modified T cells by CD28 co-stimulation will lead to antigen-induced IL2 release by CD4+ cells in situ. If this is the case, then exogenous IL2 will not need to be co-administered when T cells are modified with our anti-CEA IgTCR+IgCD28 vector. If this does not occur we will implement studies aimed at developing methods of inducing modified T cells to release IL2 after contacting CEA.

In summary, the confluence of improved T cell circulation, increasing the persistence of T cells in vivo, and inducing antigen-driven T cell proliferation, and IL2 release will allow chimeric receptor-based therapies to more closely mimic a naturally occurring immune response. It is logical to hypothesize that such a therapy will have a much higher level of anti-tumor efficacy.

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Figure 1.

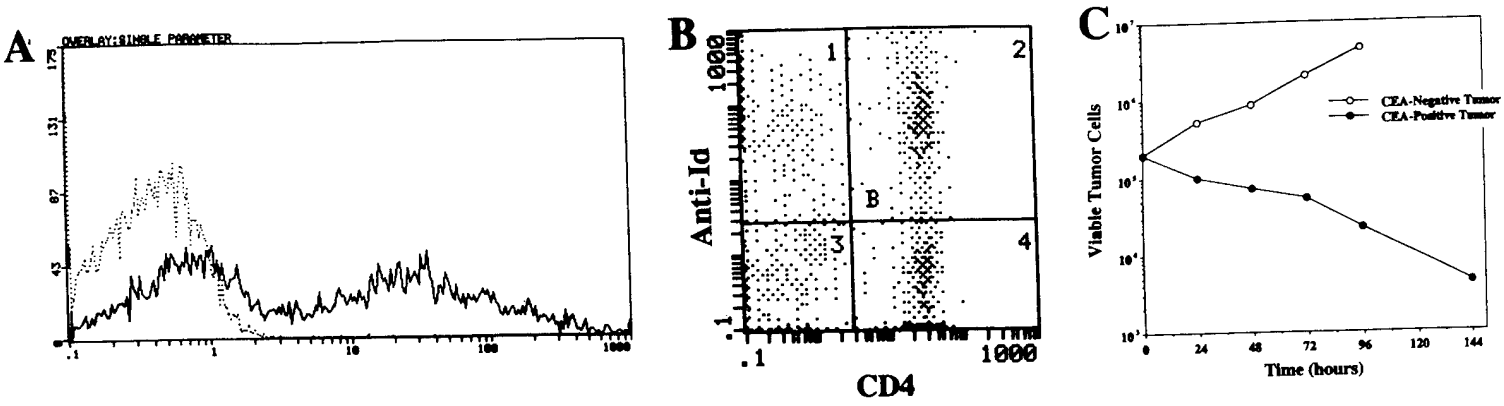

A
OVERLAY:SINGLE PARAMETER
175
131
87
43
.1
1
10
100
1000
B
Anti-Id
1000
.1
1
2
3
4
1000
CD4
C
Viable Tumor Cells
CEA-Negative Tumor
CEA-Positive Tumor
0
24
48
72
96
120
144
Time (hours)

Figure 2.

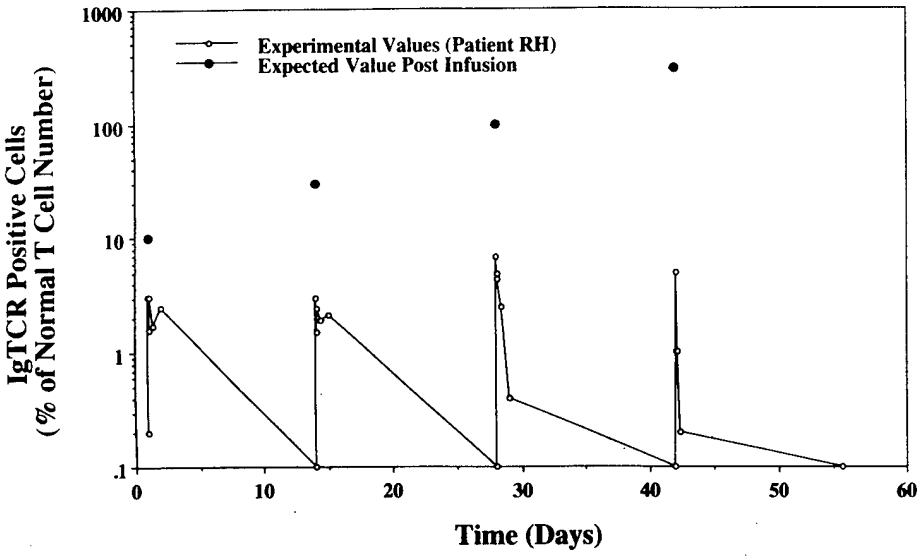

Experimental Values (Patient RH)
Expected Value Post Infusion
IgTCR Positive Cells
(% of Normal T Cell Number)
1000
100
10
1
.1
0
10
20
30
40
50
60
Time (Days)

Figure 3.

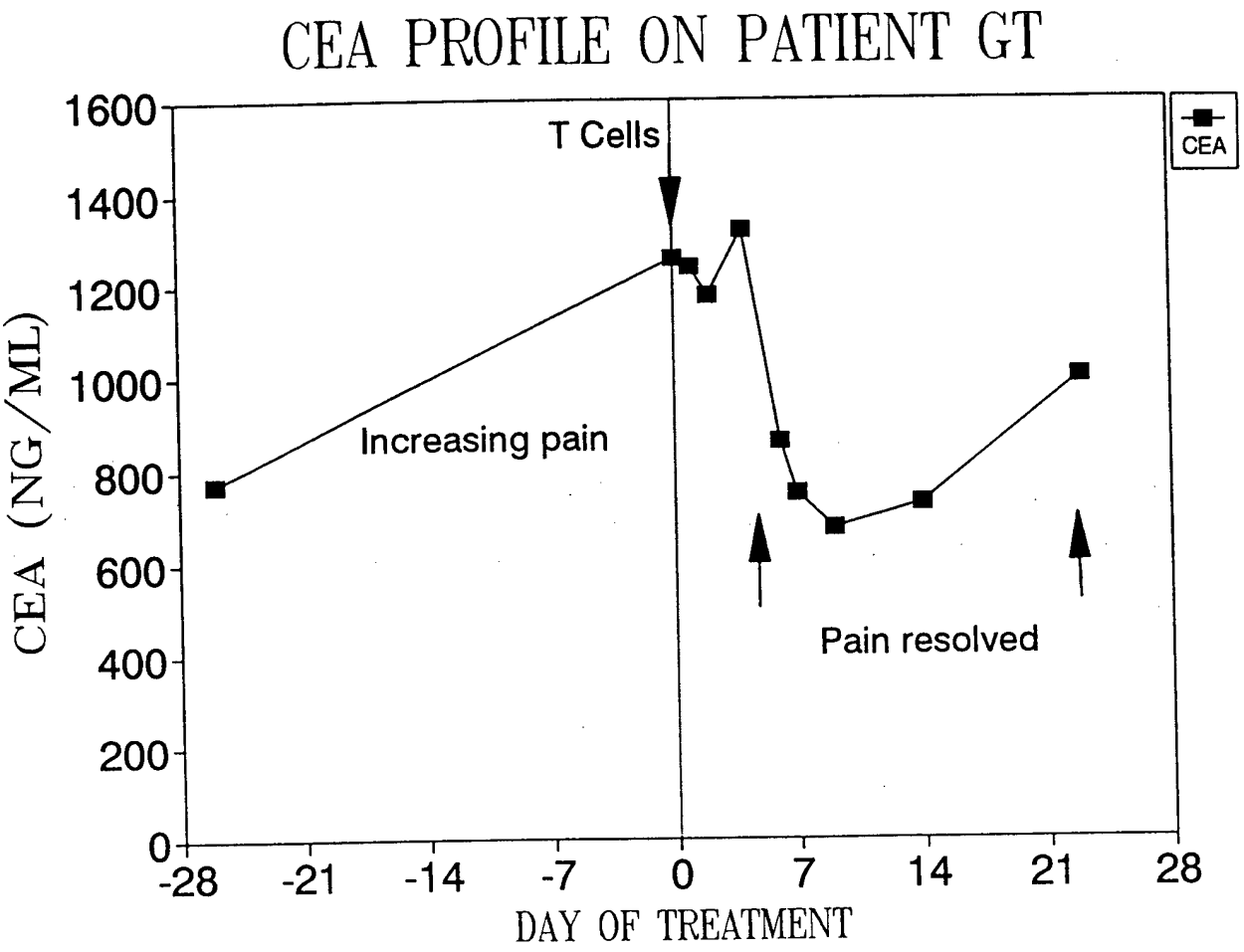

CEA PROFILE ON PATIENT GT
CEA (NG/ML)
1600
1400
1200
1000
800
600
400
200
0
T Cells
CEA
Increasing pain
Pain resolved
-28
-21
-14
-7
0
7
14
21
28
DAY OF TREATMENT

Figure 4.

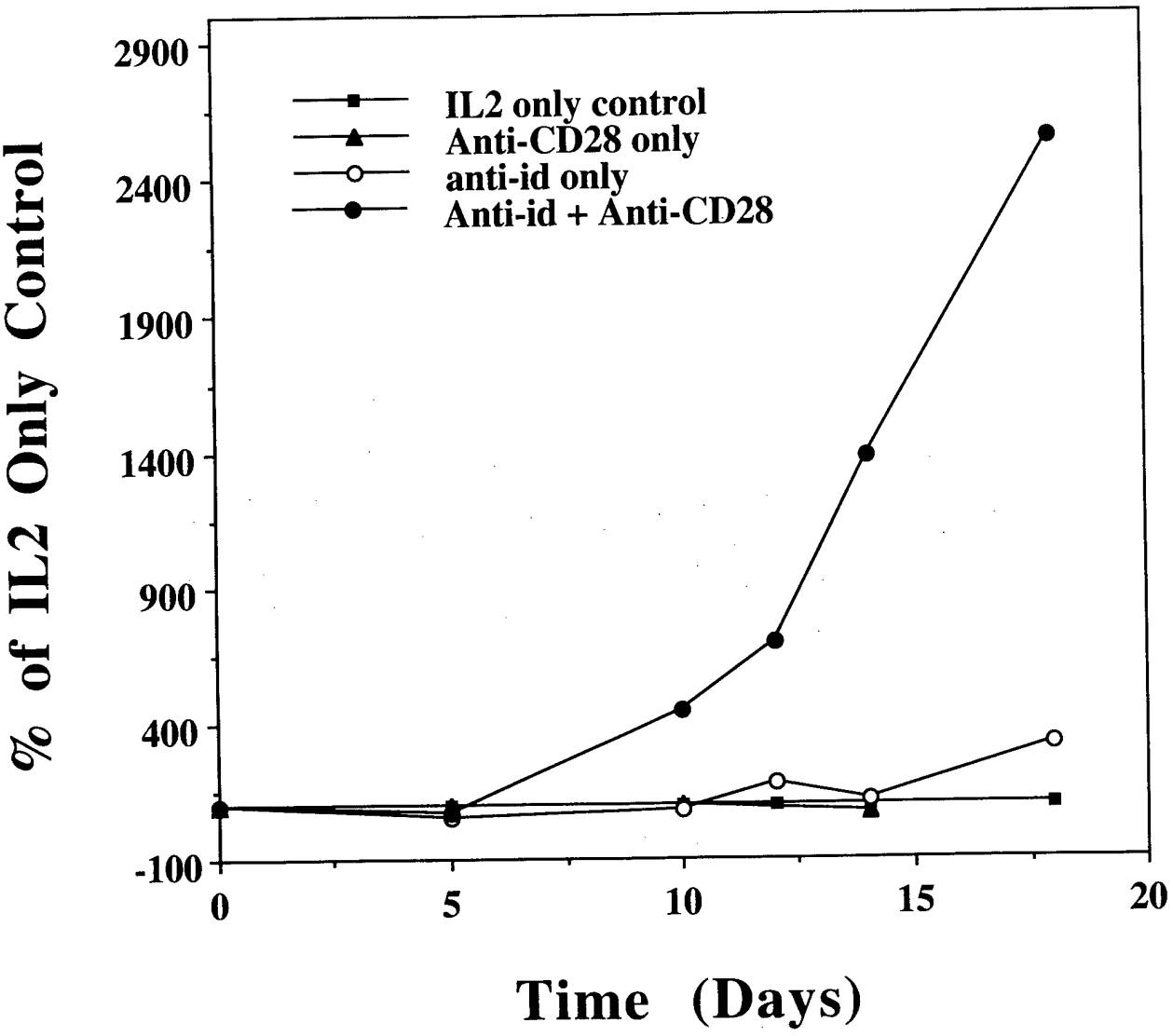

2900
2400
1900
1400
900
400
-100
0
5
10
15
20
% of IL2 Only Control
Time (Days)
IL2 only control
Anti-CD28 only
anti-id only
Anti-id + Anti-CD28

Figure 5.

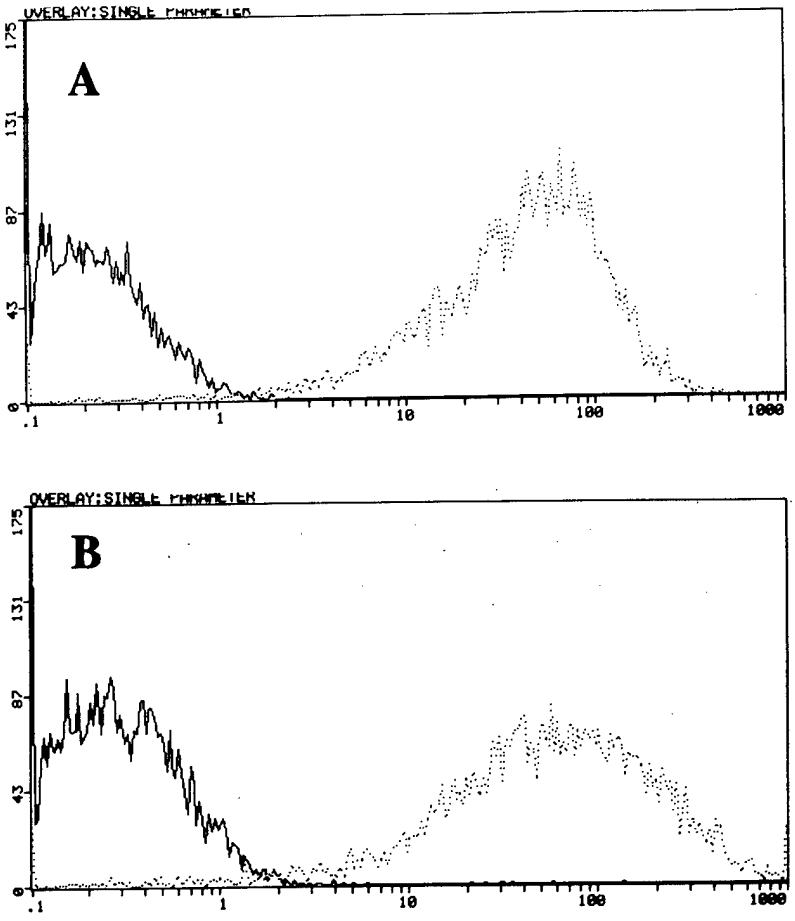


Figure 6.

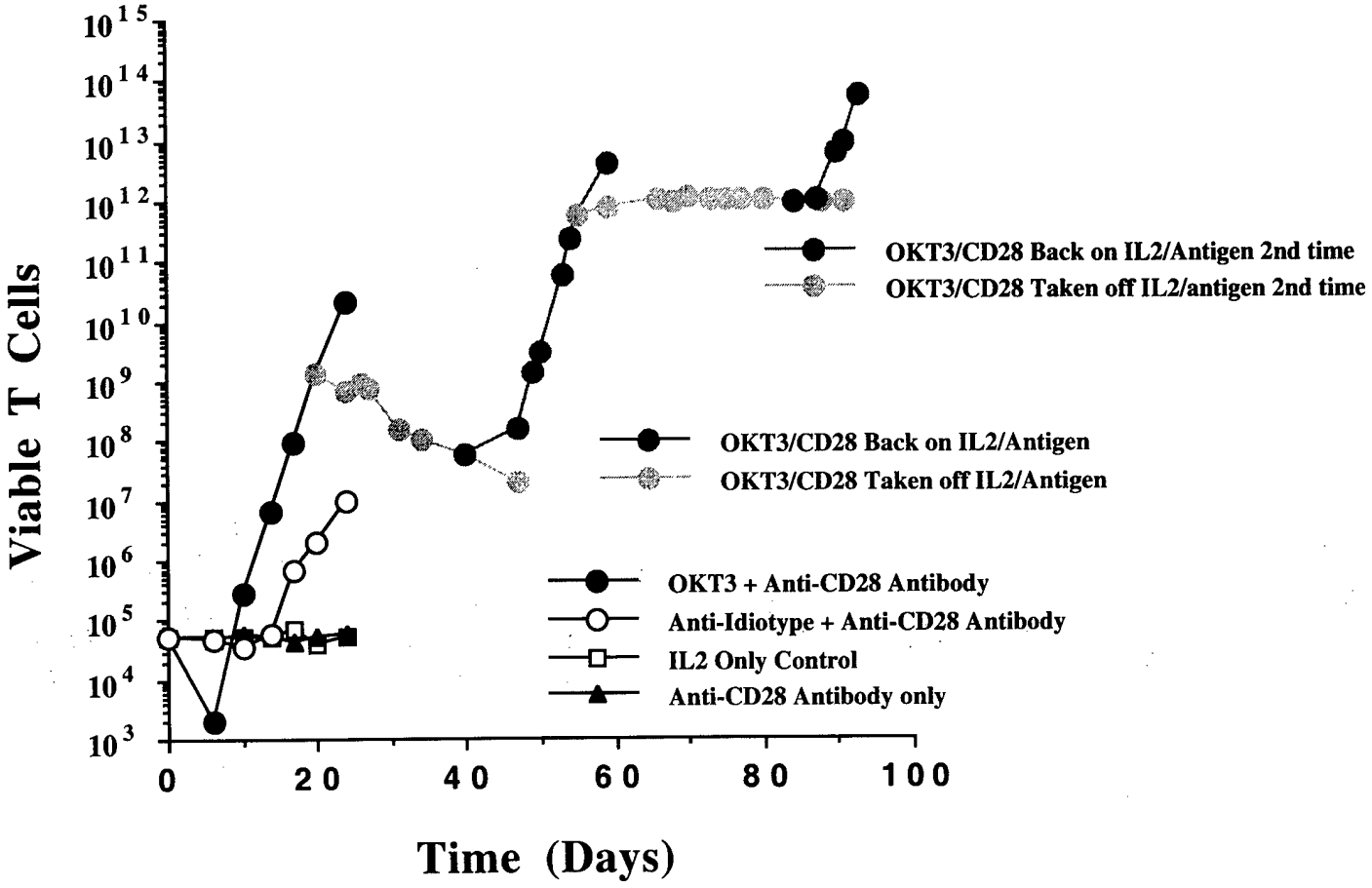
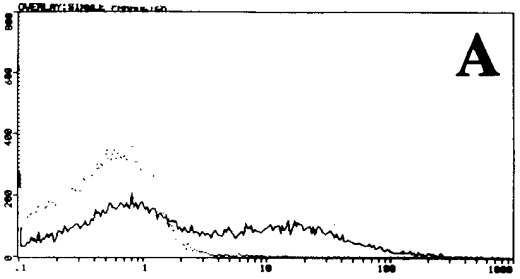
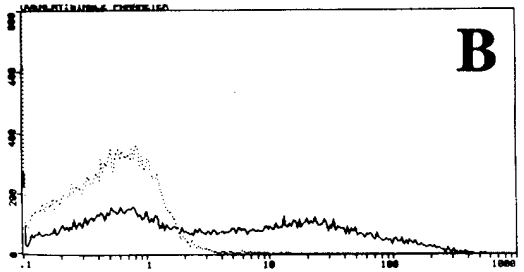

Viable T Cells
10^{15}
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
0
20
40
60
80
100
Time (Days)
OKT3/CD28 Back on IL2/Antigen 2nd time
OKT3/CD28 Taken off IL2/antigen 2nd time
OKT3/CD28 Back on IL2/Antigen
OKT3/CD28 Taken off IL2/Antigen
OKT3 + Anti-CD28 Antibody
Anti-Idiotype + Anti-CD28 Antibody
IL2 Only Control
Anti-CD28 Antibody only

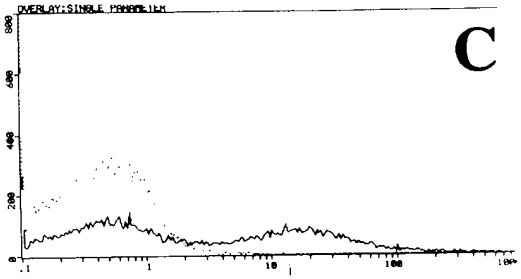
Figure 7.



Starting Population

OKT3/CD28 Selected

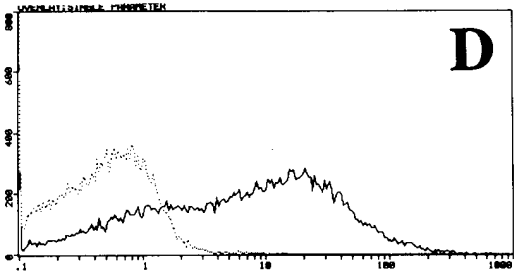




After 5 Days of Selection

After 10 Days of Selection

Anti-idiotype/CD28 Selected



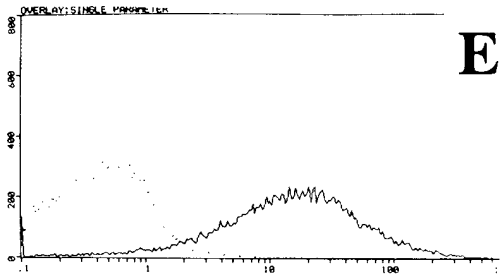


Figure 1- Representative Patient Data on Potency. *Panel A; flow cytometric analysis of IgTCR-modified patient T cells stained with negative control antibody (dotted line) and anti-idiotype antibody (solid line). Panel B; IgTCR-modified patient T cells stained with anti-idiotype antibody (Y-axis) and antibody against human CD4 (X-axis). IgTCR+CD4+ cells are in quadrant 2 and IgTCR+CD8+ cells are in quadrant 1. Panel C; dose response from tumor cell killing assays using IgTCR-modified patient T cells. Patient T cells shown in panel A. were inoculated into 6-well plates containing growing CEA+ colon carcinoma target cells at the effector to target cell ratios shown in the legend. The number of viable tumor cells at different times was determined by trypan blue exclusion and plotted as a function of time.*

Figure 2- Representative Patient Pharmacokinetic Data. *Peripheral blood lymphocytes from patient blood samples post-infusion were stained for IgTCR with anti-idiotype antibody and assayed by flow cytometry. Open circles depict the percentage of IgTCR+ T cells actually detected in patient samples while closed circles represent the percentage of IgTCR+ T cells that would theoretically be expected in circulation post infusion. Theoretical values are based on the number of T cells normally found in the circulation at any given time (e.g., $\approx 1x10^6$ T cells per milliliter of whole blood times total blood volume of 5 liters=$5x10^9$ total T cells in circulation). As an example, dose number 3 was $1x10^{10}$ T cells, of which, ≈50% were IgTCR+. The total number of IgTCR+ T cells infused is then $5x10^9$ cells, which is equal to 100% of the T cells normally found in circulation.*

Figure 3- Profile of Soluble CEA from Patient GT. *Serum samples were drawn from the patient prior to infusion of cells and at specified times after infusion. The amount of soluble CEA present in serum samples was measured using an enzyme-linked immuno assay.*

Figure 4.- Proliferation of T cells stimulated by different plate-bound antigens. *Tissue culture plates were coated with antibodies directed against the CD28 receptor (Anti-CD28 only), the IgTCR receptor (Anti-id only), with both antibodies together (Anti-id+Anti-CD28), or with no antibodies (IL2-only control). T cells from Fig. 1A were then added to the plates and incubated at 37°C. At the specified times, cells were harvested from the plates, mixed with trypan blue, and counted using a hemocytometer. Cell counts were normalized to the number of cells present in the control plates to allow averaging of data from repeat experiments. Repeated experiments used separate preparations of T cells whose rate of background replication differed slightly.*

Figure 5.- Cell surface expression of anti-CEA IgTCR and anti-CEA IgCD28 chimeric constructs. *T cells were infected with a retroviral vector containing both a chimeric IgTCR gene and a chimeric IgCD28 gene. Following infection, the cells were stained with PE-conjugated antibodies directed against the IgTCR molecule (Panel A) or the IgCD28 molecule (Panel B) and analyzed by flow cytometry. Solid lines depict control T cells that were not infected with virus, while dotted lines depict the same T cells that were infected.*

Figure 6- Isolation of rare T cell clones with a T memory cell-like phenotype. *T cells from Fig. 1A were added to 96-well tissue culture plates coated with antibodies directed against the normal T cell receptor and the CD28 receptor (OKT3+Anti-CD28), the IgTCR and CD28 receptors (Anti-idiotype+Anti-CD28), the CD28 receptor alone (Anti-CD28 only), or with no antibodies (IL2 only control). The plates were then incubated at 37°C in AIMV media containing 300 U/ml of IL2. At specific times, wells from each treatment group were harvested, mixed with trypan blue, and the number of viable cells determined by counting on a hemocytometer. On day 20, an aliquot of the cells stimulated with OKT3 and anti-CD28 were removed, washed with AIMV, and resuspended in AIMV media without IL2. These cells were then counted as above over a period of 20 days. On day 40, these IL2-rested T cells were recovered from the minus-IL2 plates, and re-cultured in plates that contained the OKT3 and anti-CD28 antibodies as well as 300 U/ml of IL2. These cells were then counted as above for an additional 20 days. The withdrawal of IL2 and antigens was then repeated on day 58. Lastly, T cells were returned to plates containing IL2 and antigen on day 83. T cell counts during periods of IL2/antigen withdrawal are shown with light grey symbols. Cell counts during periods of IL2/antigen re-stimulation are shown with dark grey symbols.*

Figure 7- Stimulating mixed populations of IgTCR and IgTCR- cells with anti-idiotype and anti-CD28 antibodies leads to the selective expansion of only IgTCR+ cells. *Tissue culture plates were coated with antibodies directed against the normal T cell receptor and the CD28 receptor (OKT3/CD28 selected),or the IgTCR and CD28 receptors (Anti-idiotype/CD28 selected). T cells transduced with a vector containing the anti-CEA IgTCR gene were then incubated at 37°C in these antigen-coated plates. The cells were stained for IgTCR expression on day zero (Panel A, starting population), and after 5 and 10 days of antigen/IL2 stimulation. Panels B and C show the staining profiles for OKT3/CD28-stimulated cells on day 5 and 10, respectively. Panels D and E show the staining profiles for anti-idiotype/CD28-stimulated cells on day 5 and 10, respectively. Dotted lines represent control T cells that were not infected with vector, while solid lines depict T cells that were infected with vector.*

Proliferation of IgTCR+ T cells after stimulation with different plate-bound antigens. *T cells from Fig.1 A were cultured in IL2-containing media until the cells reached the plateau phase of their growth curve. A fixed number of T cells were then added to tissue culture plates coated with the different antigens shown in the figure legend. Proliferation values are plotted as percent of control cells (IL2 only), which received no antigenic stimulation.*

DYNAMICS OF TUMOR CELL KILLING BY HUMAN T LYMPHOCYTES ARMED WITH AN ANTI-CEA CHIMERIC IMMUNOGLOBULIN T CELL RECEPTOR

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Tumor cell killing by anti-CEA IgTCR+ T cells

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Abbreviations used: CEA, carcinoembryonic antigen; CTL, cytotoxic T lymphocyte; LAK, lymphokine activated killer cell

Abstract

Chimeric immunoglobulin T cell receptors (IgTCR) join the antigen binding portion of an antibody to one of the signalling chains of the T cell receptor. We previously reported on the construction and functional testing of an IgTCR gene directed against the CEA tumor antigen. These preclinical studies showed the proper assembly and cell surface expression of anti-CEA IgTCR molecules, as well as specific target antigen binding and activation of T cell effector functions. Although IgTCR-modified T cells function well in vitro, therapeutic applications in humans may be complicated by a variety of factors such as the availability of appropriate T cell cytokines, high systemic levels of antagonistic soluble CEA, and antigenic diversity in tumor cell populations. In the present study we have made a detailed analysis of tumor cell killing by IgTCR-modified human T cells under conditions that more closely model those that may be encountered in human cancer patients. Specifically, we show that : 1) depriving IgTCR-modified T cells of IL2 does not diminish anti-CEA CTL activity, but does eliminate LAK-mediated tumor cell killing, 2) high levels of soluble CEA do not significantly inhibit tumor cell killing even when ≈80% of the chimeric receptors are blocked, and 3) CEA+ tumor cells that are able to downregulate cell surface CEA evade immune destruction by IgTCR-modified T cells. These results have important implications on application strategies and protocol design considerations for early clinical testing of IgTCR anti-tumor therapies.

Key words: Immunoglobulin T cell receptor
Cancer gene therapy
Adoptive immunotherapy
Carcinoembryonic antigen
Cytotoxic T lymphocyte
Lymphokine activated killer cell

INTRODUCTION

Adoptive cellular immunotherapy in cancer treatment involves the transfer of cultured immune cells such as lymphokine activated killer (LAK) cells or tumor infiltrating lymphocytes (TILs) into cancer patients. Although LAK and TIL therapies have shown some therapeutic responses, primarily in melanoma and renal cell cancer (1), their overall usefulness has been limited by a lack of tumor specificity by LAKs, and by the difficulty in isolating TILs that are not functionally tolerized to the tumor. To overcome these problems, investigators have attempted to augment cellular immunity by exploiting the high specificity of monoclonal antibodies. Initially, bispecific antibodies were conceived as a means to crosslink specific antigens on tumor cells with activation molecules on T or NK cells. However, this approach is dependent upon the diffusion and binding interactions between three separate species (e.g., tumor cell, bispecific antibody, and T cell) which is often problematic in vivo, particularly in the case of solid tumors. A more recent evolution of this concept has been to incorporate the antibody molecule directly into the T cell receptor through the construction of chimeric immunoglobulin T cell receptors (IgTCR). IgTCRs consist of the binding portion of an antibody, either as an Fab or sFv fragment, fused to one of the resident chains of the T cell receptor complex.

Several laboratories have examined the structure and function of IgTCR molecules in vitro (2-14). These studies have shown that activation of T cells through the IgTCR molecule leads to the release of cytokines such as IL2 (3, 4, 6, 8, 14), γINF (10), GM-CSF (7, 11), and TNFα (3, 11). The majority of these studies have shown that IgTCR-modified effector

cells are capable of redirected, antigen-specific cytolysis of target cells (4-14). Two studies (8, 9) have shown that gene-modified T cells will proliferate in response to activation signals delivered through the IgTCR molecule. In vivo studies have been limited but have established the anti-tumor activity of IgTCR-modified effector cells in tumor-bearing animals (10, 15)

The use of IgTCR-modified T cells in cancer treatment has several advantages, as well as several possible drawbacks. One advantage is that the chimeric receptor molecule circumvents tolerization by providing T cells with a receptor molecule that can specifically recognize and bind to a given tumor antigen. A second advantage is that the chimeric receptor allows T cell activation to occur through a direct interaction with the tumor cell. This eliminates the need for antigen to be presented by accessory cells and allows IgTCR-modified T cells to activate their effector functions independently of MHC class I or class II antigen presentation. Lastly, activation through the chimeric receptor occurs irrespective of the context of MHC class I expression. This removes the ability of tumor cells to avoid immune recognition by down regulating the expression of MHC class I molecules (16).

One potential drawback to the use of T cells armed with chimeric IgTCR genes is that circumventing the normal T cell activation pathway may also bypass the recruitment of immune helper functions provided by various accessory cell populations. Thus, the normal system of "help" and cytokine expression that supports a naturally occurring immune response may be unavailable to IgTCR-modified cells infused into patients. The availability

of IL2 is crucial for the growth and viability of IgTCR-modified T cells. IL2 secretion following antigen binding has been shown in transformed T cell lines such as Jurkat cells (8, 18), the MD45 mouse hybridoma line (3, 6, 14), and mouse EL4 cells (4). However, no study has yet shown that IgTCR+ T cells derived from normal blood will secrete IL2 after binding the target antigen. In the absence of exogenously added IL2, IgTCR-modified T cells may lack anti-tumor activity.

A second potential drawback is that soluble forms of the targeted tumor antigen will also bind to the chimeric receptor and these soluble ligand molecules may act as an antagonist. The concentration of soluble tumor antigen can reach very high levels in cancer patients, and it is unknown what effect this will have on the gene-modified T cells or on their anti-tumor efficacy in vivo. Lastly, any variant clones of the tumor that do not express the targeted tumor antigen will be able to evade immune destruction by IgTCR-modified T cells. It is likely that all of these problem areas will need to be overcome before IgTCR-modified T cells can be truly effective anti-cancer agents in human patients.

We previously reported (17, 18) on the construction and functional testing of several different IgTCR molecules directed against the carcinoembryonic tumor antigen (CEA). We showed the proper assembly and cell surface expression of anti-CEA IgTCR molecules, as well as specific target antigen binding and activation of T cell effector functions following tumor cell binding (IL2 release and tumor-specific cytolysis). In the present study we have made a more systematic analysis of tumor cell killing by IgTCR-modified human T cells

under conditions that more closely model those that we expect to encounter in human cancer patients. Specifically, we have determined; 1) the effect of IL2-deprivation on tumor cell killing, 2) the relative resistance of anti-tumor activity to high levels of soluble CEA, and 3) the potential of antigen-negative variant tumor cells to evade immune destruction. The results are discussed in the context of application strategies for clinical testing of IgTCR anti-tumor therapies.

MATERIALS AND METHODS

Retroviral Vector and Vector Producer Cells

The construction of anti-CEA IgTCR genes has been previously described (17). Based on equivalency in human T cell activation tests and ease of expression, we selected a construct containing a sFv antibody fragment fused to the ζ chain of the TCR for clinical development. This expression cassette contains sequences encoding the heavy and light chain variable regions (joined by a flexible linker) from the humanized MN14 antibody (17) fused to sequences encoding the ζ-chain of the human T cell receptor. The antibody and ζ-chain sequences are separated by the hinge region of CD8α. The clinical retroviral vector was constructed by subcloning the 1.4-kb anti-CEAsFvζ expression cassette into the NcoI-BamHI sites of the MFG vector backbone (provided by Dr. Richard Mulligan, Harvard Medical School, Boston, MA). The transgene cassette is inserted such that the initiation codon of the inserted sequences is placed precisely at the position of the viral env initiation codon. The

retroviral vector was designed to contain no selectable marker and no internal regulatory elements. Retroviral vector producer cells were constructed by transfecting the vector into the GP+E86 ecotropic helper cell line and using the transient viral supernatant to infect PG-13 cells. Viral supernatant from PG-13 cells was then used to transduce normal human PBLs.

Antibodies and Flow cytometry

The humanized MN14 antibody and its anti-idiotype antibody, WI2 (19), were obtained from Immunomedics (Morris Plains, New Jersey). hMN14 was used in the construction of the chimeric receptor as well as to detect CEA expression on tumor cells. WI2 was used to detect expression of the anti-CEA IgTCR construct, to select for anti-CEA IgTCR-positive T cells, and as a binding analog of CEA in experiments employing plate bound antigen. OKT3 (Ortho) is a mouse antibody directed against the normal human T cell receptor, and was used as a positive control staining antibody as well as to activate human T cells. UPC-10 (Sigma, St. Louis, Missouri) is an IgG2a mouse antibody with binding specificity for β-2-6 linked fructosan. UPC-10 was used as a negative control staining antibody in experiments utilizing mouse antibodies. HAT (Hoffmann-La Roche Inc., Nutley, New Jersey) is a humanized IgG1,k anti-Tac antibody and was used as a negative control staining antibody for experiments utilizing hMN14. FITC and P-phycoerythrin labeled antibodies against different human T cell antigens (CD4, CD8, CD16) and against mouse and human Fc were obtained from Caltag Laboratories (Burlingame, California). All antibody staining reactions were performed using standard methods (20). Fluorescence intensity was measured using a Coulter

EPICS Profile II flow cytometer.

Cell Lines and Culture Conditions

MIP-101 is a poorly differentiated human colorectal cancer cell line that does not express CEA (21). The MIP-CEA cell line was derived by transfecting MIP-101 with a full length cDNA encoding the human CEA gene (22). Both tumor cell lines were cultured in DMEM supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100U/ml streptomycin sulfate, and 2 mM L-glutamine.

Lymphocyte transduction and Culture

Peripheral blood mononuclear cells (PBMC) from normal blood were isolated by centrifugation over Histopaque-1083 (Sigma, St. Louis, MO). T cells were activated by culturing cells for 48 hours in AIMV media (Gibco, Gaithersburg MD) supplemented with 100 U/ml IL2, and 20 ng/ml OKT3. Activated T cells were transduced in anti-CEA IgTCR retroviral supernatant containing 10 μg/ml protamine sulfate (Fujisawa USA, Inc., Deerfield, IL) centrifuged at 1050 RCF for 1 hour at 32°C. PBMCs were transduced a total of 2-3 times. Although cell types other than T cells may be transduced at this stage, these "contaminating" cells are not stimulated to replicate under the culture conditions used, whereas treatment with OKT3 induces rapid T cell proliferation. This selective T cell proliferation quickly leads to cultures that are virtually 100% T cell in origin and effectively eliminates the influence of any "contaminating" cells from subsequent assays.

Following transduction, cells were cultured for 2 days and then selected for anti-CEA IgTCR expression by binding anti-CEA IgTCR-positive cells to tissue culture plates coated with anti-idiotype antibody (positive-panning) at either 37°C or 4°C for 30 minutes. Plates were coated by overnight incubation at room temperature with 0.1M sodium bicarbonate buffer containing 5 μg/ml of antibody. Unbound T cells were removed from the plates by gently washing the plates 2-3 times with fresh media. Adherent cells were then collected, expanded in T cell growth media and stained with PE-labeled WI2 and either FITC-labeled mouse anti-human CD4 or mouse anti-human CD8 antibodies. The percentage of anti-CEA IgTCR modified cells and the CD4:CD8 ratio was then determined by flow cytometry.

Cytotoxicity Assays

Tumor cell targets MIP-CEA (CEA-positive) and MIP-101 (CEA-negative) were plated into 6-well tissue culture plates at a density of $1x10^5$ cells/ml. After 24 hours, T cells transduced with the anti-CEA IgTCR vector were added to the plates at specific effector to target cell ratios (E:T ratio), and the plates returned to incubation at 37°C. Every 24 hours, a well from each of the different E:T ratios was harvested for counting by first washing to remove unbound T cells, adding 0.5 ml of trypsin to each well to detach the tumor cells, adding 0.5 ml of media (1 ml final volume) and then pipetting the final volume to create single cell suspensions. Cells were then diluted in media containing trypan blue and the number of viable cells counted using a hemocytometer.

Both MIP-CEA and MIP-101 cells are 2-3 fold larger than human T cells and are readily distinguished from the few residual T cells that had not been removed by the pre-trypsinization washes. The wash volumes used to remove unbound T cells were microscopically inspected to ensure that live tumor cells were not also detached from the plates. Although the vast majority of cells in the wash volumes were either T cells or dead tumor cells, a very small number of live tumor cells could be detected. Since both tumor lines are strongly adherent, these cells most likely arise from the small fraction of cells undergoing mitotic detachment at the time of washing. Due to their small numbers, live tumor cells contained in the wash volumes were not included in the cell counts. This was validated in our initial experiments by including live tumor cells from the wash volumes in the total cell counts, and demonstrating that either including or, excluding the wash volume cell counts did not significantly alter the slope of the kill curves (data not shown). In subsequent experiments, wells that were to be harvested for counting were only microscopically inspected to ensure that significant numbers of viable, detached tumor cells were not present. Invariably, the only wells in which noticeable numbers of viable, detached tumor cells were observed was after day 4-5 in wells where little or no tumor cell killing occured (e.g. untreated controls, and MIP-101 cells) . This was due mainly to the fact that in these wells, tumor cells proliferate unabated and become heavily confluent by the fourth and fifth days of the assay. Even in these cases however, the exclusion of floating cells from the final cell counts had little effect on the overall slope of the growth curves (for example, see slight decrease in slope of untreated controls from day 4 to day 5 in Fig. 3).

For all cytotoxicity assays, individual wells were counted from 2 to 9 times depending on the cell density. For wells that contained a high density of cells, a minimum of 2 duplicate counts were performed. In cases where extensive cell death made it difficult to obtain >100 cells for counting, a minimum of 9 counts were done. These cell counts were then averaged to obtain the total number of cells remaining for each time point and treatment group in a given experiment. Cell counts from repeated independent experiments were then averaged for each time point/treatment group and the number of surviving tumor cells plotted as a function of time.

RESULTS

Stable, long term expression of the IgTCR vector in normal human T cells

Cultures of primary human T lymphocytes were transduced with the anti-CEA IgTCR vector and assayed for IgTCR expression. Following 2-3 rounds of transduction, approximately 40-50% of the T cells express the anti-CEA IgTCR gene (Fig.1A). Cells that did not bind to the anti-idiotype coated plates did not express the anti-CEA IgTCR protein (Fig.1B). Gene-modified T cells were selected by binding the cells to tissue culture plates coated with anti-idiotype antibody (Fig.1C). 100% of the panned cells were able to rebind anti-idiotype coated plates (Fig.1D), indicating that the low staining fraction of cells in Fig.1C express physiologically relevant levels of the chimeric protein. T cells that were bound a second time to anti-idiotype coated plates (Fig.1D) showed a similar pattern of staining as those that were bound only once. This indicates that additional rounds of panning at 37°C did not further increase the level of anti-CEA IgTCR expression (compare Fig.1C and 1D), and that one round of panning fully selects for anti-CEA IgTCR+ T cells.

The level of anti-CEA IgTCR expression in the selected population of cells was sensitive to temperature. Panning cells at 4°C failed to bind low-expressing cells, resulting in a selection bias for cells that expressed higher levels of anti-CEA IgTCR protein (compare Fig.1C and 1E). At 4°C, the T cells assume a uniformly round shape with reduced membrane fluidity. This rounded shape presents a reduced area of the membrane to the plate surface. Cells that express quantitatively higher densities of receptors per square micron have a selective

advantage in remaining bound to the plates during washes. Hence, panning at 4°C yields populations of cells with higher levels of IgTCR expression. At 37°C, the cells have normal membrane fluidity and progressively flatten out on the bottom of the plates as more receptors surrounding the initial contact point bind immobilized antigen. This large area of binding facilitates the engagement of sufficient receptors on cells with low densities of receptors to allow them to remain bound to the plates during washes.

Both CD4+ and CD8+ T cells are modified with the anti-CEA IgTCR gene

T cells from Fig.1C above were stained with antibodies against the CD4 or CD8 receptors, and either WI2-PE or UPC-PE and then analyzed by two color flow cytometry (Fig.2). CD4+ and CD8+ T cells were transduced at roughly equal proportions with the IgTCR vector.

Expression of the anti-CEA IgTCR gene was stable in long term cultures of primary human T cells, remaining virtually unchanged over a period of >2 months (data not shown). The ratio of CD4+ to CD8+ T cells was also stable over time, although in cultures carried for >2 months the proportion of CD8+ cells slowly increased (data not shown). All T cell cytotoxicity experiments described in this report were performed using populations of T cells that were 100% positive for expression of the anti-CEA IgTCR gene, and contained CD4:CD8 ratios of ≈60:40. Populations of T cells were selected for anti-CEA IgTCR expression by anti-idiotype panning at 37°C as shown above.

Potent, targeted killing of CEA-positive tumor cells

In the present study, tumor cell killing was measured using a trypan-blue based assay. This assay allows tumor cell killing to be measured cumulatively over a period of several days, and is thus more sensitive than ^{51}Cr release assays. This increased sensitivity allows the use of much lower effector-to-target (E:T) cell ratios. In comparison to ^{51}Cr release assays, this assay has a more directly interpretable meaning in terms of net tumor cell killing. A comparison of cytotoxicity data using both the trypan-blue based, and a ^{51}Cr release assay demonstrated that the trypan blue assay had greater sensitivity, was more reproducible, and did not suffer from the high background counts associated with the leakage of ^{51}Cr from untreated cells (data not shown). Other more minor validation studies are described in the materials and methods section.

Cytotoxicity at different E:T ratios was determined by co-incubating anti-CEA IgTCR-modified T cells (from Fig.1C) with either the CEA-negative cell line, MIP-101, or with CEA-positive MIP-CEA cells. The number of viable tumor cells in each treatment group were counted by trypan blue exclusion and plotted as a function of incubation time (Fig.3). At an E:T ratio of 0.5:1, MIP-CEA cells were progressively destroyed over a period of several days (Fig.3A). At higher E:T ratios, the elimination of MIP-CEA tumor cells was accelerated in a dose dependent manner.

In contrast to MIP-CEA, MIP-101 cells continued to proliferate when incubated with anti-CEA IgTCR+ T cells at low E:T ratios (Fig.3B), although their growth rate was

reproducibly lower than untreated controls. Non-specific killing of MIP-101 cells occurred at all E:T ratios above 2:1. Although this toxicity was significantly less than that against MIP-CEA cells, it nonetheless occurred in a dose-dependent manner. Depletion of any residual natural killer cells from the T cell cultures by negative-panning with anti-CD16 antibody did not eliminate this non-specific cytotoxicity (data not shown).

T cells retain the ability to lyse CEA-positive tumor cells in the absence of IL2

A primary goal of the present studies was to examine anti-CEA IgTCR mediated cytolysis under conditions similar to those that might occur *in vivo* in human clinical trials. The availability of IL2 in vivo could play a significant role in supporting the ability of IgTCR-modified T cells to activate anti-tumor effector functions. IL2 and other cytokines are secreted by CD4+ gene-modified cells in response to binding target antigen (3, 4, 6-11, 14, 18). However, it is uncertain whether local secretion will be sufficient to maintain T cell viability and activity. To determine the importance of the continued presence of IL2, we examined the effect of IL2 withdrawal on anti-CEA IgTCR-mediated cytotoxicity.

T cells were prepared by culturing separate aliquots of cells from Fig.1C in media that contained either 100 U/ml of IL2 or no exogenously added IL2. Approximately 70% of the T cells die within 7-10 days after removal of IL2 from the cultures, as shown by the high proportion of cells with low forward scatter (Fig.4A). Dead cells were removed from the cultures by centrifugation over Histopaque-1083 and the remaining live cells were used in tumor cell killing assays. In IL2-deprived cultures, the surviving T cells changed in

morphology from an activated appearance (large, irregularly shaped cells) to a resting appearance (small, homogeneously round cells) (Fig.4A). In addition, the expression of the anti-CEA IgTCR gene decreased significantly in IL2-rested cultures (Fig.4B), paralleling the generalized downregulation of protein synthesis, including actin and normal TCR proteins (X.Y. Tan and R.P. Junghans, unpublished data). Despite these changes, IL2-rested T cells retain their ability to kill MIP-CEA tumor cells (Fig.5A). Furthermore, the non-specific toxicity previously observed against MIP-101 target cells was completely eliminated (Fig.5B). Even at very high E:T ratios, where all MIP-CEA cells are lysed within 24 hours (Fig.6A), MIP-101 target cells incubated with IL2-rested T cells proliferate as rapidly as untreated controls (Fig.6B). Control assays using T cells that remained in IL2 media, and done in parallel with the experiments shown in Fig.5A and B, gave similar rates of killing at each E:T ratio (data not shown). Thus, differences in the rate of killing between Fig. 3, Fig. 5, Fig. 6 are due to inter-assay variability.

As mentioned above, the removal of NK cells from the cultures by negative-panning on CD16 coated plates did not eliminate non-specific toxicity against MIP-101 cells, whereas removing IL2 from the cultures did. We next sought to determine if the non-specific toxicity against MIP-101 cells resulted from the activation state of the T cells or from the elimination of a specific subset of T cells that were selected against by IL2 deprivation. T cell cultures rested as in Figure 4 were returned to IL2-containing media for 2-3 days. These IL2-rescued T cells recovered their activated appearance (Fig.4A) and high anti-CEA IgTCR expression (Fig.4B). Non-specific cytotoxicity also reappeared in these IL2-rescued T cells (Fig.5C).

Subsequent experiments showed that removal of IL2 from the cultures one hour prior to the co-incubation step was sufficient to eliminate non-specific cytotoxicity to MIP-101 (data not shown). No T cell death occurs after only one hour of IL2 deprivation.

High levels of soluble CEA do not prevent anti-CEA IgTCR-mediated cytotoxicity

An important question is what effect high concentrations of soluble CEA will have on the kinetics of anti-CEA IgTCR-mediated tumor cell killing. To determine the effect of soluble CEA, T cells were incubated with MIP-CEA cells in the presence of 1000 ng/ml and 10,000 ng/ml of soluble CEA (Fig.7, panels A and B respectively). Anti-CEA IgTCR modified T cells efficiently killed MIP-CEA tumor cells in the presence of both 1000 and 10,000 ng/ml of soluble CEA. In some of the kill curves, the rate of cytolysis was slightly slower in the presence of soluble CEA than in its absence, but even in these instances soluble CEA did not substantially block tumor cell killing.

Immune evasion by tumor cells that have down-regulated cell surface CEA

At very low E:T ratios (<0.1 E:T), a large number of CEA-positive tumor cells are killed by anti-CEA IgTCR-modified T cells. Despite this, a fraction of the tumor cells escape T cell killing and eventually go on to overgrow the cultures (data not shown). These surviving tumor cells grow as distinct colonies, indicating that survival was the result of either random immune escape due to the sparse seeding of T cells, or a specific selection of tumor cell variants that are resistance to anti-CEA killing. To determine if colony formation was due to specific evasion of anti-CEA T cells, surviving tumor cells were expanded in culture and

then analyzed for cell surface CEA expression. These oligoclonal MIP-CEA survivors were stained with hMN14 antibody and their cell surface CEA levels compared to those of MIP-CEA cells that had not been exposed to anti-CEA IgTCR T cells (Fig.8). As expected, the parental MIP-101 and MIP-CEA tumor cells showed no deviation from their characteristic CEA expression profiles (Fig.8A and 8B, respectively). However, the oligoclonal MIP-CEA survivors from the cytotoxicity assay (Fig.8C) included a preponderance of cells with lower cell surface CEA protein. The majority of these survivor cells have no detectable CEA, while a smaller fraction ($\approx$20%), retain CEA expression that is slightly lower than the parental MIP-CEA cell line. This latter group may be a result of stochastic escape in which a fraction of CEA-positive tumor cells escape containment by the sparsely seeded T cells.

DISCUSSION

Expression of the IgTCR vector in normal human T cells

To facilitate long-term engraftment of the vector-modified cells, we designed the anti-CEA IgTCR vector to contain virtually no foreign gene products. This was accomplished by using a humanized antibody to derive the immunoglobulin portion of the chimeric molecule and human sequences for the hinge/TCR portions of the chimera (18). Furthermore, no microbial drug selection marker was included in the vector. To overcome the lack of drug selection, we substituted positive-panning of transduced cells on anti-idiotype-coated plates to select gene-modified T cells. As shown in Fig.1, this method of selection can yield

preparations of cells that are 100% positive for the transgene in as rapidly as 30 minutes. An added advantage of this method is that selection is based on expression of the therapeutic transgene itself rather than on an irrelevant marker gene.

Targeted Killing of CEA-positive Tumor Cells

From a therapeutic standpoint, the most important functional test of anti-CEA IgTCR-modified T cells is their ability to redirect cytolytic functions and specifically lyse CEA-positive tumor cells. At an E:T ratio of 0.5:1, less than 4% of the CEA+ tumor cells remained viable after 5 days, while CEA- tumor cells expanded $\approx$15-fold over the same period. Approximately 40% of these T cells are CD8+ cells which yields a CTL:tumor cell ratio of 0.2:1. The elimination of tumor cells at this low effector to target cell ratio confirms that modified T cells recycle their lytic capacity to kill multiple targets, as in normal T cell killing. Given the sparseness of the initial T cell seeding ($2x10^5$ cells per well of 6-well plate), this also indicates that the modified T cells are mobile on the plate in finding the targets, which are themselves adherent. Similar experiments utilizing PBMCs isolated from several colon carcinoma patients showed no significant differences in CEA-directed cytolysis from PBMCs derived from normal donors (data not shown). The independent roles of CD4+ and CD8+ T cells in tumor cell killing is currently under investigation.

Non specific killing of CEA-negative tumor cells was found to be dependent upon IL2, suggesting that this toxicity was mediated by T-LAK cells (23, 24). T-LAKs are lymphokine

activated killer cells that are derived from T cell precursors. T-LAK cells are characterized by their ability to lyse a wide spectrum of tumor targets and can develop cytotoxic activity in the absence of any antigenic stimulation (23, 24). Although there are conflicting results on the origins of LAK precursors and their characteristic phenotypes as effector cells (25, 26, 27, 28, 29), it is clear that LAK cells can be derived from either NK or T cell precursors.

Antigen-specific killing is preserved in the absence of IL2, but T-LAK activity is eliminated.

In vivo levels of IL2 in cancer patients treated with IgTCR-modified T cells may play a critical role in regulating anti-tumor efficacy. Although CD4+ IgTCR-modified T cells secrete IL2 after binding antigen (18), it is not known if this is sufficient to support tumor cell killing in the absence of exogenously added IL2. As such, we sought to determine the effect of IL2-deprivation on anti-tumor activity. As expected, depriving the T cells of IL2 led to apoptosis and a marked decrease in viability. T cells that survived IL2 withdrawal converted to a deactivated phenotype (Fig. 4). Despite this general deactivation, IL2-rested T cells retained the ability to respond to CEA+ tumor cells and were undiminished, on a per cell basis, in their ability to lyse CEA+ tumor cells (Fig. 5). Likewise, the low levels of IgTCR protein expressed in IL2-rested cells (Fig. 4B) did not significantly reduce CTL activity. This is similar to the results shown in Fig. 1E in which low IgTCR expression did not block the ability to bind antigen (Fig. 1E), and indicates that IgTCR expression that is below that detectable by FACS, is nonetheless physiologically functional.

Depriving T cells of IL2 also served to illuminate the two distinct mechanisms by which tumor cell killing is accomplished in this model. When exogenous IL2 is withdrawn, tumor cell killing is exclusively CEA-specific and is mediated through a CTL-like mechanism. Although IgTCR-mediated killing is not MHC-dependent, as in normal CTL killing, it is antigen-dependent and utilizes a similar signalling process. Withdrawing IL2 just prior to the co-incubation step was sufficient to eliminate all non-specific toxicity (data not shown), indicating that IL2 is the key mediator of this non-specific toxicity. Even at very high doses of T cells (Fig.6A and B), tumor cytolysis is entirely antigen-specific in the absence of IL2, whereas in the presence of IL2, there is a moderate level of non-specific T-LAK activity against MIP-101 cells (data not shown).

In the presence of exogenous IL2, tumor cell killing appears to occur through a combination of non-specific LAK-like activity (T-LAK), and antigen-specific CTL-like activity. LAK cells lyse a wide spectrum of target cells independently of any antigenic stimulation (23, 24), while CTLs only lyse cells that display the target antigen. The results show that non-specific T-LAK-mediated killing is active only in the presence of IL2 (Fig.5C). Comparing the proportion of killing attributable to each type of mechanism shows that CTL-type killing is far more potent than LAK-type killing. For example, at an E:T ratio of 0.5:1, non-specific LAK type killing accounts for $\approx$1-2 fold reduction in tumor cell number (Fig.3B), while antigen specific CTL-type killing accounts for $\approx$500 fold reduction (Fig.3A).

Soluble CEA does not block Tumor Cell Killing

Many patients with CEA-expressing tumors have high serum levels of soluble CEA, which may inhibit anti-CEA IgTCR-mediated tumor cell killing. The level of soluble CEA in the serum of patients infrequently exceeds 1000 ng/ml (17), while 10 fold higher levels might be expected to occur in the immediate tumor bed microenvironment. Tumor cell killing was not blocked at either of these concentrations of soluble CEA, although there is a modest dose-dependent inhibition, that is most readily detected at the lower E:T ratios.

Expressed in terms of Kd, soluble CEA at 1000 ng/ml (5 nM) is well below the sFv Kd of 21 nM, and will not significantly reduce the number of free chimeric receptors (17). Soluble CEA at 10,000 ng/ml (50 nM) is $\approx$2.5 times the sFv Kd and will reduce the number of free receptor molecules by $\approx$80%. We previously hypothesized that soluble CEA, which can only bind monovalently to the chimeric receptor, will not be able to compete with the stabilized, polyvalent interactions that occur with immobilized CEA on the surface of tumor cells. The fact that cytotoxicity was only slightly reduced when $\approx$80% of the receptors are blocked supports this hypothesis and suggests that soluble CEA will not be able to block cytotoxicity in vivo.

Immune evasion by tumor cells that have down-regulated CEA surface expression

It is becoming clear that tumor cells can evolve a number of mechanisms to evade immune destruction, including downregulating MHC class I expression, Fas ligand-induced apoptosis of infiltrating lymphocytes (30, 31), and secreted immunosuppressive factors (e.g., TGFβ) (32). T cells redirected by chimeric IgTCR genes will not be affected by some of these

mechanisms (e.g. MHC class I repression) but may be inhibited by others.

The simplest mechanism by which tumor cells can evade anti-CEA IgTCR-modified T cells is to downregulate expression of the target antigen. The majority of tumor cells that evaded anti-CEA IgTCR-mediated killing had no FACS-detectable CEA expression, while a smaller fraction ($\approx$20%) expressed low levels of CEA. Close examination of the CEA expression profile in the parental MIP-CEA cells (Fig.8) reveals a small tail of low- or non-expressing tumor cells. These low expressing tumor cells are most likely the progenitors of the majority of surviving tumor cells. Surviving tumor cells that still express CEA may have escaped destruction by the sparse seeding and lack of contact during the assay period. In vivo, it is possible that some tumor cells will evade immune destruction by anti-CEA IgTCR-modified T cells simply by loss of CEA expression. Ultimately, it may be necessary to provide chimeric receptors to two or more tumor antigens. For example, T cells targeted to colon carcinomas could be armed with chimeric receptors to both the CEA and Tag-72 tumor antigens.

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FIGURE LEGENDS

Fig. 1- Normal human T lymphocytes transduced with the anti-CEA IgTCR vector. OKT3-activated human T cells were infected twice with the anti-CEA IgTCR vector, stained with either PE-labeled WI2 anti-idiotype antibody (solid lines) or with UPC negative control antibody (dotted lines) and then analyzed by flow cytometry. (Panel A) T cells stained with WI2 or UPC one day after the second round of infection. (Panel B) T cells from panel A that did not bind to anti-idiotype coated tissue culture plates. (Panel C) T cells from panel A selected for anti-CEA IgTCR expression by binding the cells to anti-idiotype coated tissue culture plates at 37°C. (Panel D) T cells from panel A selected for anti-CEA IgTCR expression by binding the cells to anti-idiotype coated tissue culture plates at 4°C. (Panel E) T cells from panel C that re-bound anti-idiotype coated tissue culture plates at 37°C.

Fig. 2- IgTCR-modification of both CD4+ and CD8+ T cells. The proportion of CD4+, CD8+, and IgTCR+ T cells, was determined by two-color flow cytometry. Green fluorescence (FITC channel) is plotted on the x-axis and red fluorescence (PE channel) on the y-axis. T cells from Fig.1C were stained with the following antibodies: (Panel A) mouse-anti-human CD4-FITC and negative control antibody UPC-PE. (Panel B) mouse-anti-human CD4-FITC and WI2 antidiotype anitbody. (Panel C) mouse-anti-human CD8-FITC and negative control antibody UPC-PE. (Panel D) mouse-anti-human CD8-FITC and WI2 antidiotype anitbody.

Fig. 3- Targeted killing of CEA-positive tumor cells.

CEA-positive tumor cells (MIP-CEA, Panel A) or CEA-negative tumor cells (MIP-101, Panel B) were co-incubated with anti-CEA IgTCR-modified T cells. Viable tumor cells are plotted as a function of time. Data were averaged from 5-7 individual experiments at each of the E:T ratios shown in the figure legends. Error bars denote +/- the standard deviation. *Error bars at 72 and 96 hours in the 5:1 E:T ratio go negative due to zero values in one of the repeat experiments, which cannot be graphed.

Fig. 4- IL2-dependent T cell morphological changes.

Flow cytometric analysis of human T cells after activation with OKT3 + IL2, 10 days after removal of IL2 from the cultures (IL2 rested), and 2-3 days after cells were restimulated with IL2 (IL2 re-activated). (Panel A) Flow cytometric profiles of log side scatter (LSS) versus forward scatter. Populations of live cells are shown enclosed in the circles used to gate the cells on the flow cytometer. Populations of dead and dying cells are indicated with arrows. (Panel B) Fluorescence intensity of cells stained with anti-idiotype antibody (IgTCR+).

Fig 5.- Tumor cell killing in the absence of exogenous IL2.

Anti-CEA IgTCR modified T cells were IL2 rested for 7-10 days, and dead cells were removed by centrifugation over ficoll. IL2-rested T cells were then co-incubated with MIP-CEA and MIP-101 tumor cells. (Panel A) MIP-CEA tumor cell killing in the absence of IL2. (Panel B) MIP-101 tumor cell killing in the absence of IL2. (Panel C) MIP-101 tumor cell killing by T cells cultured without IL2 for 10 days and then reactivated by culturing the cells in IL2-containing media for 2-3 days. E:T ratios used in each experiment are shown next to each graph. E:T ratios used in panel C are higher than in panels A and B in order to detect any non-specific toxicity.

Fig. 6- Complete loss of T-LAK activity with IL2-rested T cells.

IL2 rested anti-CEA IgTCR modified T cells, as in Fig.4 were co-incubated with MIP-CEA and MIP-101 tumor cells and cytoxicity was measured. (Panel A) MIP-CEA tumor cell killing in the absence of IL2. (Panel B) MIP-101 tumor cell killing in the absence of IL2. E:T ratios used in each experiment are shown next to each graph.

Fig. 7- Inhibition of tumor cell killing by soluble CEA.

Anti-CEA IgTCR modified T cells were co-incubated with MIP-CEA tumor cells in the presence and absence of soluble CEA. (Panel A) MIP-CEA tumor cell killing in the presence of 1000 ng/ml of soluble CEA. (Panel B) MIP-CEA tumor cell killing in the presence of 10,000 ng/ml of soluble CEA. E:T ratios used in each experiment are shown next to each graph.

Fig. 8- CEA-positive tumor cells that lack cell surface CEA evade immune destruction. Tumor cells surviving in culture after co-incubation with a low number of T cells (E:T, $<0.1:1$) were analyzed for surface CEA expression. Tumor cells were stained with negative control antibody (solid line) or with hMN14 antibody (dotted line). (Panel A) MIP-101 tumor cells. (Panel B) parental MIP-CEA tumor cells . (Panel C) MIP-CEA oligoclonal survivor cells from cytoxicity assays.

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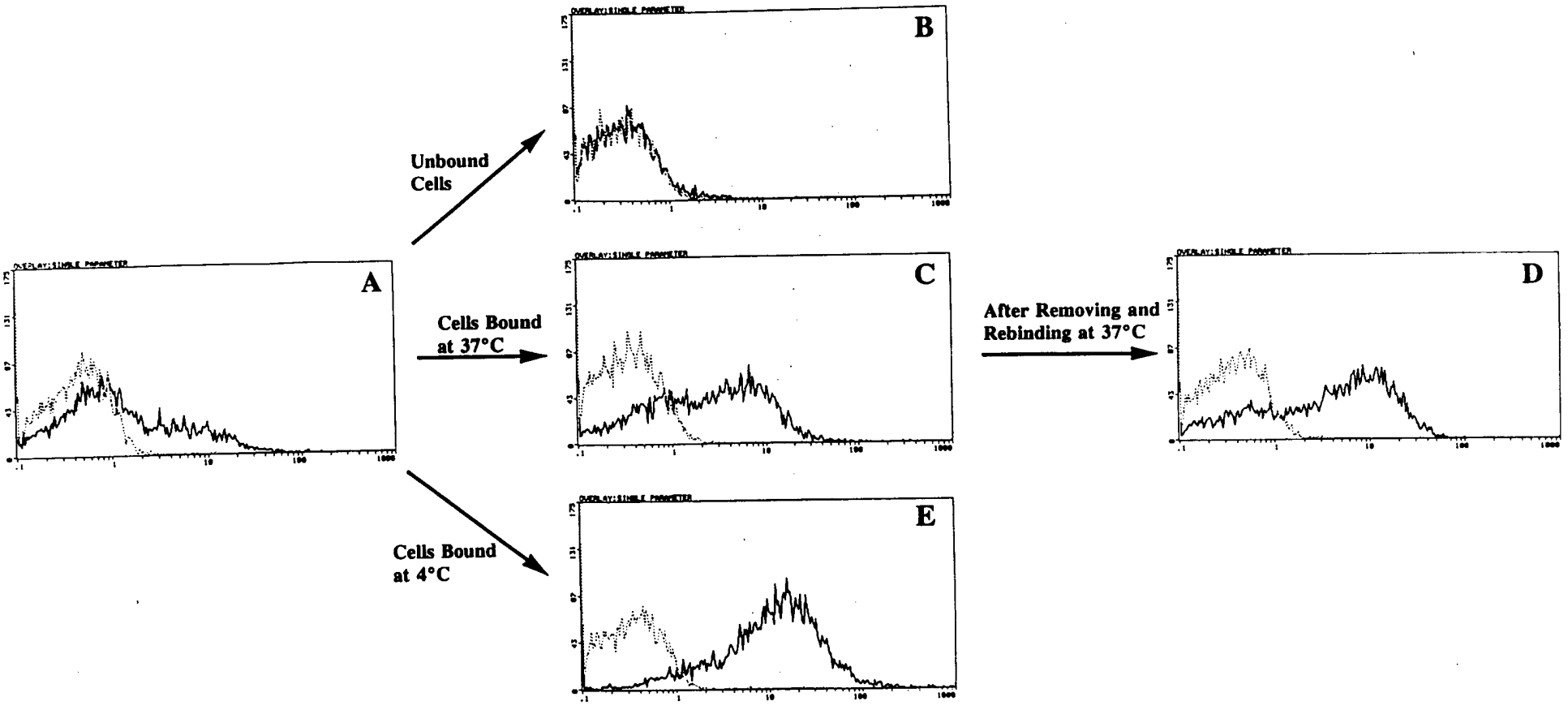
Fig 1


A
B
C
D
E
Unbound Cells
Cells Bound at 37°C
Cells Bound at 4°C
After Removing and Rebinding at 37°C

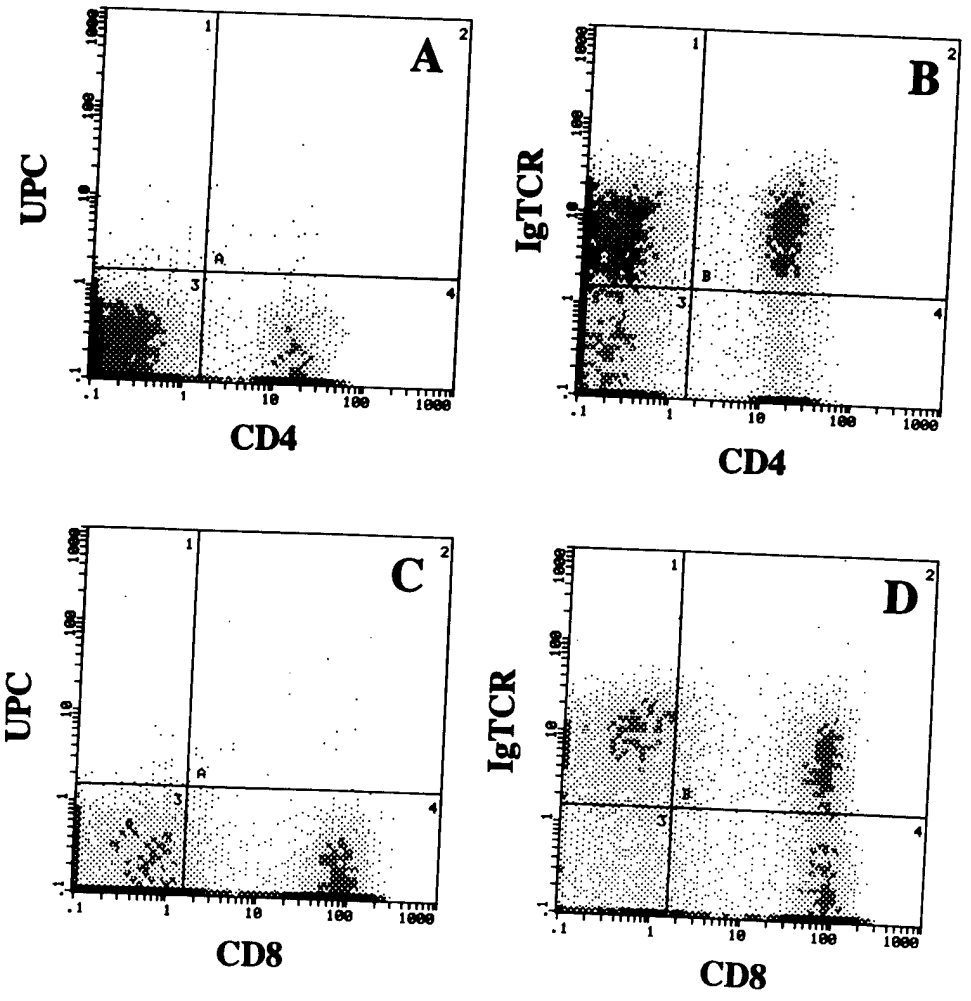
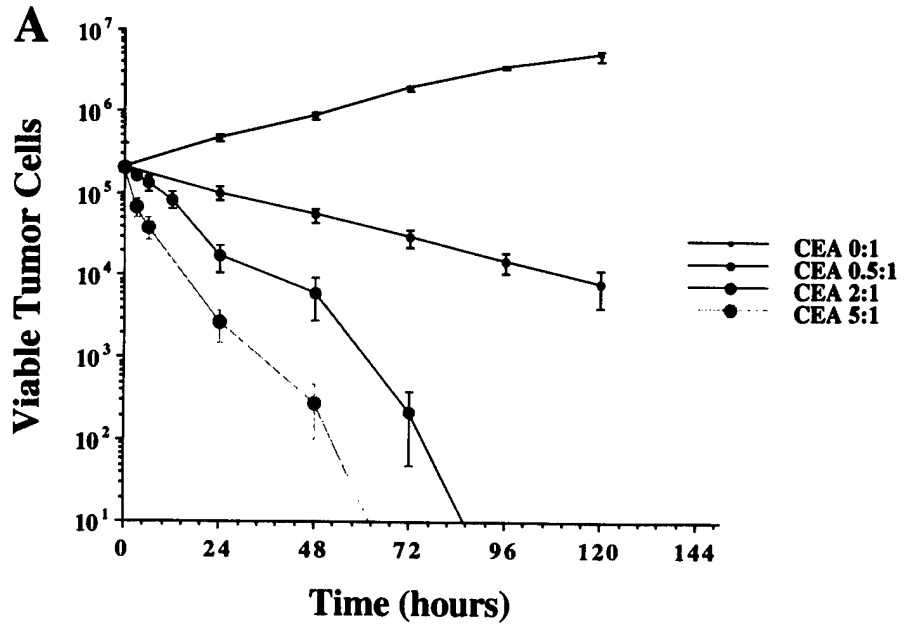


Fig 2


A
Viable Tumor Cells
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
24
48
72
96
120
144
Time (hours)
CEA 0:1
CEA 0.5:1
CEA 2:1
CEA 5:1

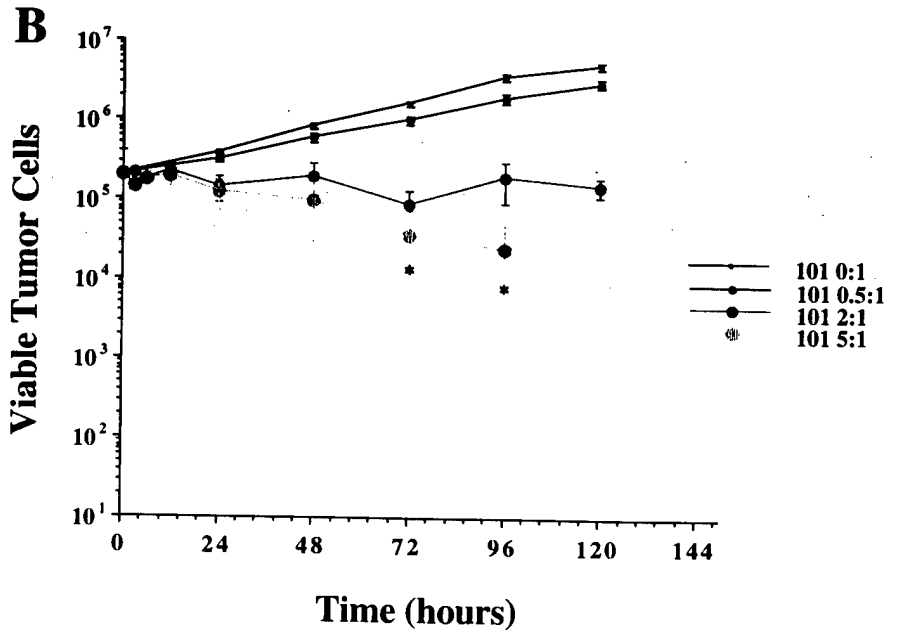
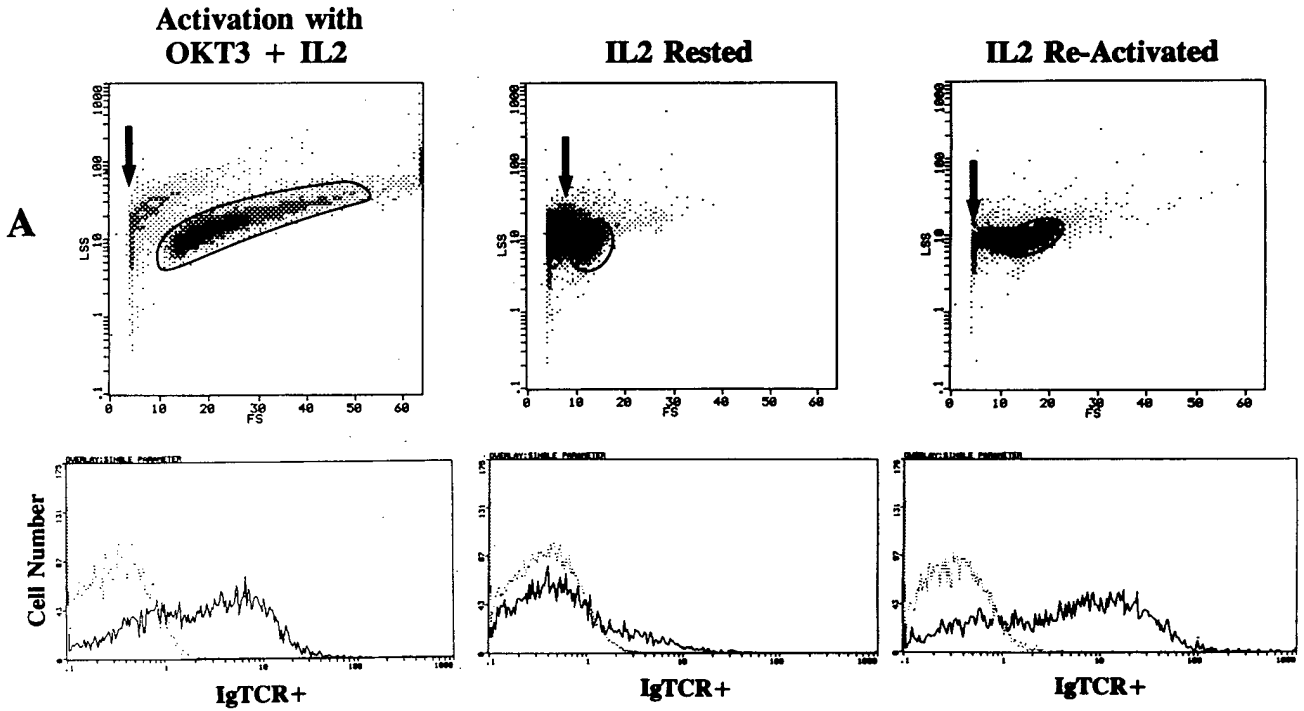
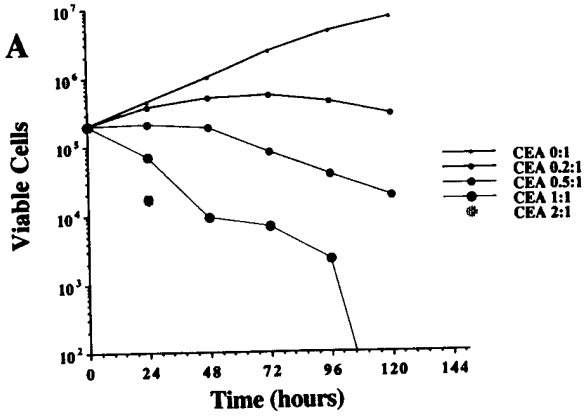
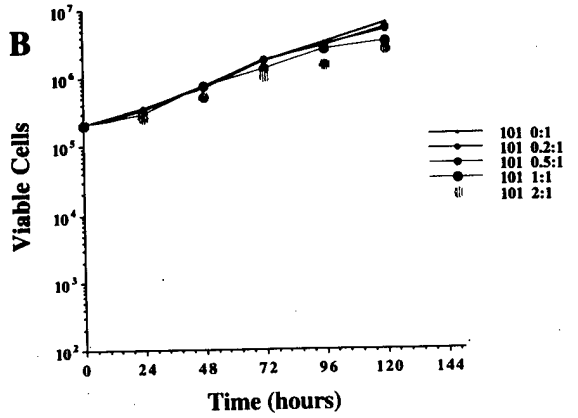

B
Viable Tumor Cells
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
24
48
72
96
120
144
Time (hours)
101 0:1
101 0.5:1
101 2:1
101 5:1

Fig 3

Fig 4


Activation with
OKT3 + IL2
IL2 Rested
IL2 Re-Activated
A
B
Cell Number
IgTCR+
IgTCR+
IgTCR+


A
Viable Cells
10^7
10^6
10^5
10^4
10^3
10^2
0
24
48
72
96
120
144
Time (hours)
CEA 0:1
CEA 0.2:1
CEA 0.5:1
CEA 1:1
CEA 2:1


B
Viable Cells
10^7
10^6
10^5
10^4
10^3
10^2
0
24
48
72
96
120
144
Time (hours)
101 0:1
101 0.2:1
101 0.5:1
101 1:1
101 2:1

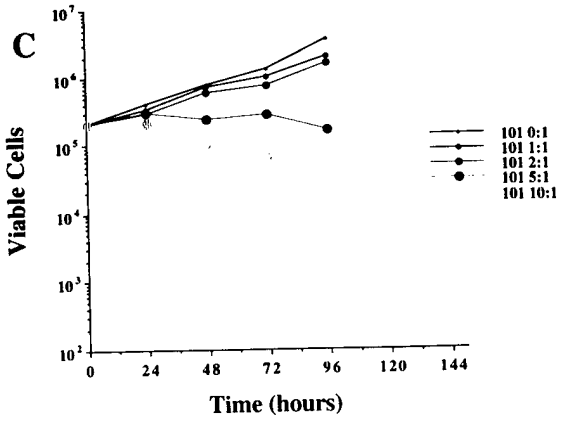

C
Viable Cells
10^7
10^6
10^5
10^4
10^3
10^2
0
24
48
72
96
120
144
Time (hours)
101 0:1
101 1:1
101 2:1
101 5:1
101 10:1

Fig 5

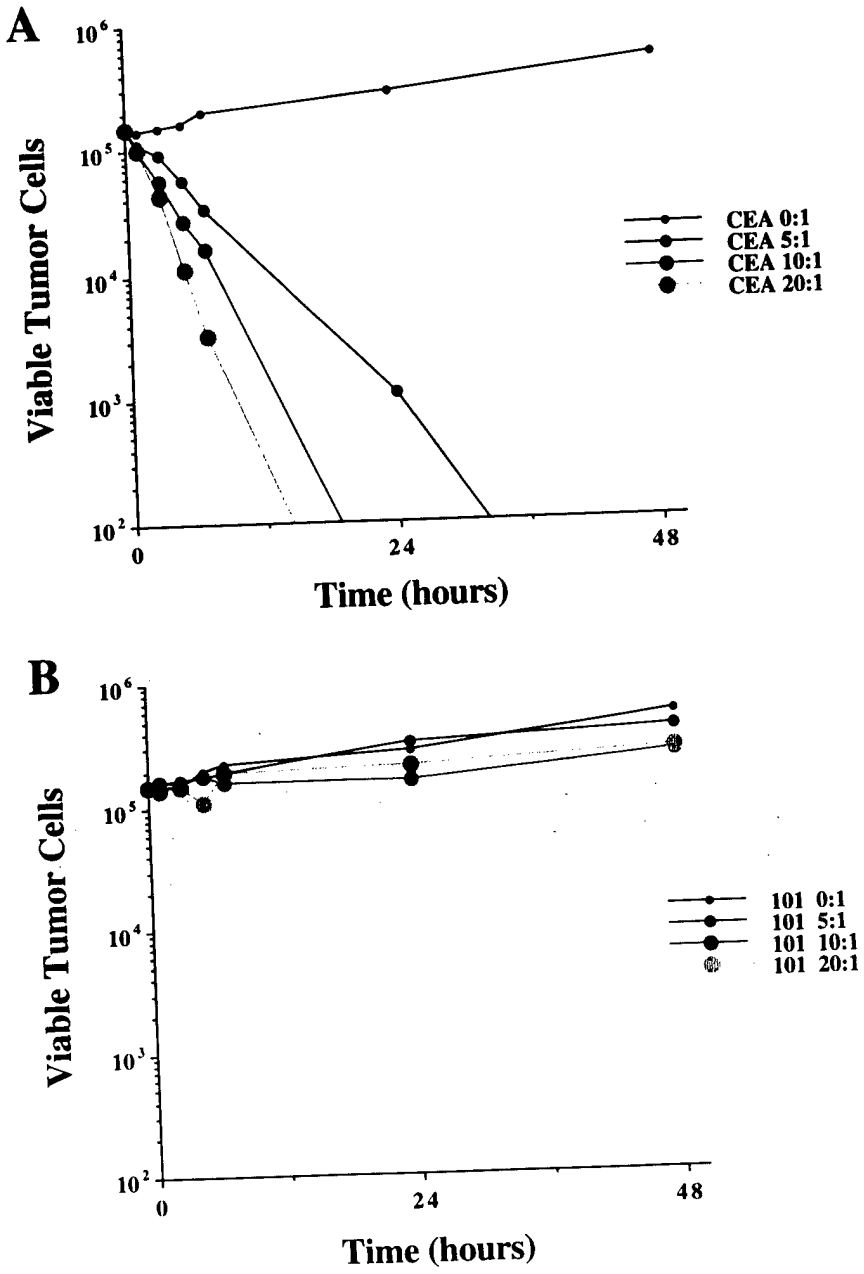

A
Viable Tumor Cells
10^6
10^5
10^4
10^3
10^2
0
24
48
Time (hours)
CEA 0:1
CEA 5:1
CEA 10:1
CEA 20:1
B
Viable Tumor Cells
10^6
10^5
10^4
10^3
10^2
0
24
48
Time (hours)
101 0:1
101 5:1
101 10:1
101 20:1

Fig 6

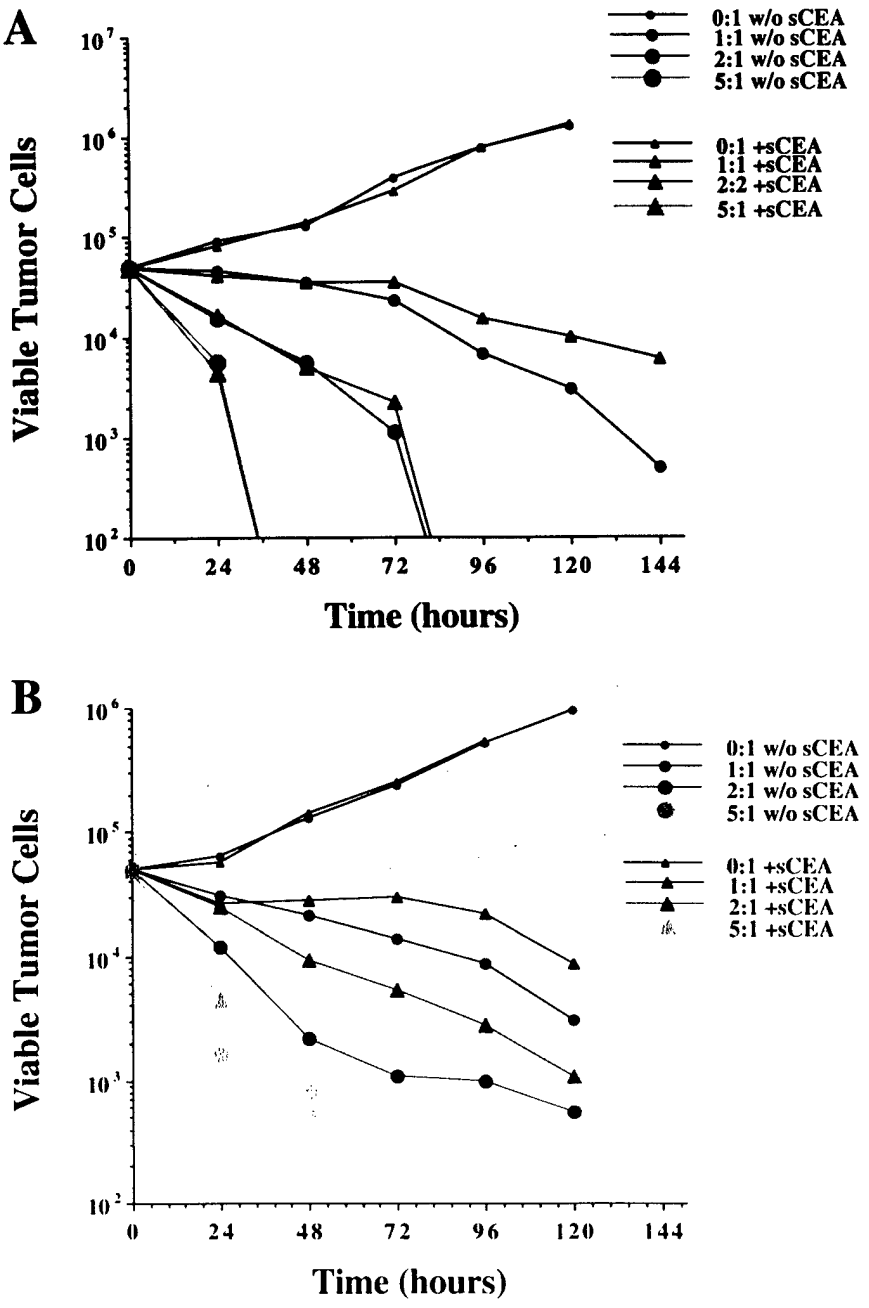

A
Viable Tumor Cells
10^7
10^6
10^5
10^4
10^3
10^2
0
24
48
72
96
120
144
Time (hours)
0:1 w/o sCEA
1:1 w/o sCEA
2:1 w/o sCEA
5:1 w/o sCEA
0:1 +sCEA
1:1 +sCEA
2:2 +sCEA
5:1 +sCEA
B
Viable Tumor Cells
10^6
10^5
10^4
10^3
10^2
0
24
48
72
96
120
144
Time (hours)
0:1 w/o sCEA
1:1 w/o sCEA
2:1 w/o sCEA
5:1 w/o sCEA
0:1 +sCEA
1:1 +sCEA
2:1 +sCEA
5:1 +sCEA

Fig 7

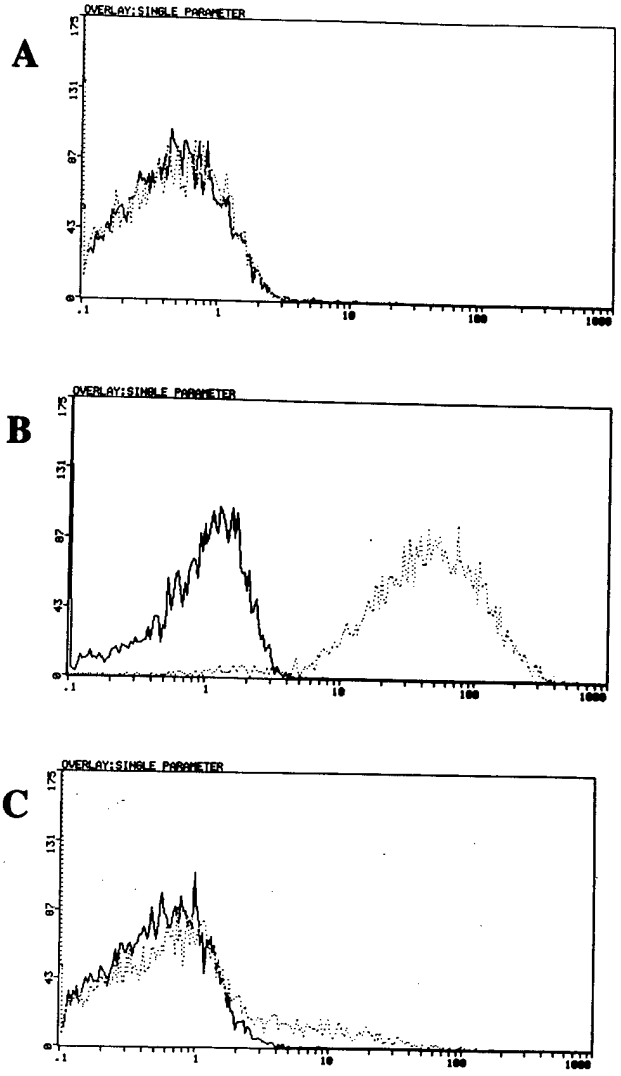

A
OVERLAY:SINGLE PARAMETER
175
131
87
43
0
.1
1
10
100
1000
B
OVERLAY:SINGLE PARAMETER
175
131
87
43
0
.1
1
10
100
1000
C
OVERLAY:SINGLE PARAMETER
175
131
87
43
0
.1
1
10
100
1000

Fig 3